POLYMORPHIC MARKERS OF PROSTATE CARCINOMA TUMOR ANTIGEN –1 (PCTA-1)

RELATED APPLICATIONS

The present application claims priority from U.S. Provisional Patent Application Serial No. 60/088,187, filed June 5, 1998, and U.S. Provisional Patent Application Serial No. 60/102,324, filed September 28, 1998, the disclosures of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The invention concerns the genomic and cDNA sequences of the *PCTA-1* gene, biallelic markers of the *PCTA-1* gene and the association established between these markers and prostate cancer. The invention provides means to determine the predisposition of individuals to prostate cancer as well as means for the diagnosis of this cancer and for the prognosis/detection of an eventual treatment response to therapeutic agents acting against prostate cancer.

BACKGROUND OF THE INVENTION

Prostate Cancer

The incidence of prostate cancer has dramatically increased over the last decades. It averages 30-50/100,000 males in Western European countries as well as within the US White male population. In these countries, it has recently become the most commonly diagnosed malignancy, being one of every four cancers diagnosed in American males. Prostate cancer's incidence is very much population specific, since it varies from 2/100,000 in China, to over 80/100,000 among African-American males.

In France, the incidence of prostate cancer is 35/100,000 males and it is increasing by 10/100,000 per decade. Mortality due to prostate cancer is also growing accordingly. It is the second cause of cancer death among French males, and the first one among French males aged over 70. This makes prostate cancer a serious burden in terms of public health.

Prostate cancer is a latent disease. Many men carry prostate cancer cells without overt signs of disease. Autopsies of individuals dying of other causes show prostate cancer cells in 30 % of men at age 50 and in 60 % of men at age 80. Furthermore, prostate cancer can take up to 10 years to kill a patient after the initial diagnosis.

The progression of the disease usually goes from a well-defined mass within the prostate to a breakdown and invasion of the lateral margins of the prostate, followed by metastasis to regional lymph nodes, and metastasis to the bone marrow. Cancer metastasis to bone is common and often associated with uncontrollable pain.

Unfortunately, in 80 % of cases, diagnosis of prostate cancer is established when the disease has already metastasized to the bones. Of special interest is the observation that prostate cancers frequently grow more rapidly in sites of metastasis than within the prostate itself.

Early-stage diagnosis of prostate cancer mainly relies today on Prostate Specific Antigen (PSA) dosage, and allows the detection of prostate cancer seven years before clinical symptoms become apparent. The effectiveness of PSA dosage diagnosis is however limited, due to its inability to discriminate between malignant and non-malignant affections of the organ and because not all prostate cancers give rise to an elevated serum PSA concentration. Furthermore, PSA dosage and other currently available approaches such as physical examination, tissue biopsy and bone scans are of limited value in predicting disease progression.

Therefore, there is a strong need for a reliable diagnostic procedure which would enable a more systematic early-stage prostate cancer prognosis.

Although an early-stage prostate cancer prognosis is important, the possibility of measuring the period of time during which treatment can be deferred is also interesting as currently available medicaments are expensive and generate important adverse effects. However, the aggressiveness of prostate tumors varies widely. Some tumors are relatively aggressive, doubling every six months whereas others are slow-growing, doubling once every five years. In fact, the majority of prostate cancers grow relatively slowly and never becomes clinically manifest. Very often, affected patients are among the elderly and die from another disease before prostate cancer actually develops. Thus, a significant question in treating prostate carcinoma is how to discriminate between tumors that will progress and those that will not progress during the expected lifetime of the patient.

Hence, there is also a strong need for detection means which may be used to evaluate the aggressiveness or the development potential of prostate cancer tumors once diagnosed.

Furthermore, at the present time, there is no means to predict prostate cancer susceptibility. It would also be very beneficial to detect individual susceptibility to prostate cancer. This could allow preventive treatment and a careful follow up of the development of the tumor.

A further consequence of the slow growth rate of prostate cancer is that few cancer cells are actively dividing at any one time, rendering prostate cancer generally resistant to radiation

and chemotherapy. Surgery is the mainstay of treatment but it is largely ineffective and removes the ejaculatory ducts, resulting in impotence. Oral oestrogens and luteinizing releasing hormone analogs are also used for treatment of prostate cancer. These hormonal treatments provide marked improvement for many patients, but they only provide temporary relief. Indeed, most of these cancers soon relapse with the development of hormone-resistant tumor cells and the oestrogen treatment can lead to serious cardiovascular complications. Consequently, there is a strong need for preventive and curative treatment of prostate cancer.

Efficacy/tolerance prognosis could be precious in prostate cancer therapy. Indeed, hormonal therapy, the main treatment currently available, presents important side effects. The use of chemotherapy is limited because of the small number of patients with chemosensitive tumors. Furthermore the age profile of the prostate cancer patient and intolerance to chemotherapy make the systematic use of this treatment very difficult.

Therefore, a valuable assessment of the eventual efficacy of a medicament to be administered to a prostate cancer patient as well as the patient's eventual tolerance to it may allow the benefit/risk ratio of prostate cancer treatment to be enhanced.

Prostate Carcinoma Tumor Antigen –1 (PCTA-1)

WO 96/21671 describes a new protein, named PCTA-1. The document describes the cloning and sequencing of a cDNA encoding PCTA-1 (GenBank L78132). This cDNA has 3.85 kb in length and presents about 80 % sequence homology with rat galectin-8.

WO 96/21671 mentions that the PCTA-1 protein retains a number of conserved structural motifs that are found in most members of the galectin gene family. On the basis of its predicted amino acid sequence, PCTA-1 is said to appear to be a human homologue of rat galectin-8. The galectins display wide tissue distribution, clear developmental regulation, and differential levels in specific tissues, supporting the hypothesis that they contribute to many physiologically important processes in mammalian cells. Of direct relevance to cancer is the finding that the galectins can mediate both cell-cell and cell-matrix interactions.

SUMMARY OF THE INVENTION

The inventors have characterized the genomic sequence of the *PCTA-1* gene, including its regulatory regions, and, through an association study, have shown that alleles of some biallelic markers of *PCTA-1* are associated with prostate cancer.

Therefore, the present invention concerns the identification and characterization of the genomic sequence of the *PCTA-1* gene, of new cDNA sequences and the proteins encoded by these cDNAs. The invention also concerns biallelic markers located in such sequences, as well as the selection of significant polymorphisms associated with prostate cancer.

Oligonucleotide probes and primers hybridizing specifically with a genomic sequence of *PCTA-1* are also part of the invention. A further object of the invention consists of recombinant vectors comprising any of the nucleic acid sequences described in the present invention, and in particular of recombinant vectors comprising the regulatory region of *PCTA-1* or a sequence encoding a PCTA-1 protein, as well as cell hosts comprising said nucleic acid sequences or recombinant vectors.

The selected polymorphisms are used in the design of assays for the reliable detection of genetic susceptibility to prostate cancer, of an early onset of prostate cancer, of the aggressiveness of prostate cancer tumors, of a modified or forthcoming expression of the *PCTA-1* gene, of a modified or forthcoming production of the PCTA-1 protein, or of the production of a modified PCTA-1 protein. They can be used for diagnosis, staging, prognosis, and monitoring of such a disease, which processes can be further included within treatment approaches. The selected polymorphisms can also be used in the design of drug screening protocols to provide an accurate and efficient evaluation of the therapeutic and side-effect potential of new or already existing medicaments.

The invention also encompasses methods of screening of molecules which modulate or inhibit the expression of the *PCTA-1* gene and more preferably of agent acting against prostate cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a diagram of the *PCTA-1* gene with an indication of the relative position of the biallelic markers of the present invention. The upper line refers to the genomic sequence of *PCTA-1*. The middle line refers to the alternative cDNA comprising the exon 6bis with the biallelic markers localization. The lower line refers the PCTA-1 protein with the polymorphic amino acids due to the biallelic markers. ✪ refers to frequent SNP (detected on pool of hundred DNA).

Figure 1B is a diagram of the 3 alternative cDNAs of *PCTA-1*.

Figure 2 is a graph demonstrating the association between some of the biallelic markers of the invention and prostate cancer with the absolute value of the logarithm (base 10) of the p-value of the chi-square values for each marker shown on the y-axis and a rough estimate of the position of each marker with respect to the *PCTA-1* gene elements on the x-axis.

Figure 3 is a block diagram of an exemplary computer system.

Figure 4 is a flow diagram illustrating one embodiment of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database.

Figure 5 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous.

Figure 6 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence.

Figures 7A-D is an alignment of the mouse and human PCTA-1 proteins.

BRIEF DESCRIPTION OF THE SEQUENCES PROVIDED IN THE SEQUENCE LISTING

SEQ ID No 1 contains a genomic sequence of *PCTA-1* comprising the 5' regulatory region (upstream untranscribed region), the exons (0, 1, 2, 3, 4, 5, 6, 6bis, 7, 8, 9, 9bis, and 9ter) and introns, and the 3' regulatory region (downstream untranscribed region).

SEQ ID No 2 contains a cDNA sequence of *PCTA-1* comprising the exons 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9.

SEQ ID No 3 contains a cDNA sequence of *PCTA-1* comprising the exons 0, 1, 2, 3, 4, 5, 6, 6bis, 7, 8, and 9.

SEQ ID No 4 contains a cDNA sequence of *PCTA-1* comprising the exons 0, 1, 2, 3, 4, 5, 6, 7, 8, 9bis and 9ter.

SEQ ID No 5 contains the amino acid sequence encoded by the cDNA of SEQ ID No 2.

SEQ ID No 6 contains the amino acid sequence encoded by the cDNA of SEQ ID No 3.

SEQ ID No 7 contains the amino acid sequence encoded by the cDNA of SEQ ID No 4.

SEQ ID No 8 contains a murine cDNA sequence of *PCTA-1.*

SEQ ID No 9 contains the amino acid sequence encoded by the cDNA of SEQ ID No 8.

SEQ ID No 10 contains a primer containing the additional PU 5' sequence described further in Example 2.

SEQ ID No 11 contains a primer containing the additional RP 5' sequence described further in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Before describing the invention in greater detail, the following definitions are set forth to illustrate and define the meaning and scope of the terms used to describe the invention herein.

The term "*PCTA-1* gene" is intended to define an entity which can comprise some or all the following elements : exons, introns, promoter, regulatory regions, 5'UTR, 3' UTR and regions never transcribed and located either upstream or downstream of the coding sequence of

PCTA-1. The term "*PCTA-1* gene", when used herein, encompasses genomic, mRNA and cDNA sequences encoding a PCTA-1 protein.

The term "heterologous protein", when used herein, is intended to designate any protein or polypeptide other than the PCTA-1 protein. More particularly, the heterologous protein is a compound which can be used as a marker in further experiments with a *PCTA-1* regulatory region or as a toxin to certain cells in which it is intended to be produced, preferably a toxin to prostate cancer cells.

As used herein, the term "toxin gene" refers to a polynucleotide sequence which encodes a polypeptide that, when expressed in a eukaryotic cell, typically a mammalian cell, kills or disables the cell or causes the cell to exhibit apoptosis, cytostasis or senescence.

The term "isolated" requires that the material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that the vector or composition is not part of its natural environment.

The term "purified" does not require absolute purity; rather, it is intended as a relative definition. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. As an example, purification from 0.1 % concentration to 10 % concentration is two orders of magnitude. The term "purified" is used herein to describe a polynucleotide or polynucleotide vector of the invention which has been separated from other compounds including, but not limited to other nucleic acids, carbohydrates, lipids and proteins (such as the enzymes used in the synthesis of the polynucleotide), or the separation of covalently closed polynucleotides from linear polynucleotides. A polynucleotide is substantially pure when at least about 50%, preferably 60 to 75% of a sample exhibits a single polynucleotide sequence and conformation (linear versus covalently closed). A substantially pure polynucleotide typically comprises about 50%, preferably 60 to 90% weight/weight of a nucleic acid sample, more usually about 95%, and preferably is over about 99% pure. Polynucleotide purity or homogeneity is indicated by a number of means well known in the art, such as agarose or polyacrylamide gel electrophoresis of a sample, followed by visualizing a single polynucleotide band upon staining the gel. For certain purposes higher resolution can be provided by using HPLC or other means well known in the art.

As used interchangeably herein, the terms "nucleic acids", "oligonucleotides", and "polynucleotides" include RNA, DNA, or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form. The term "nucleotide" as used herein as an adjective to describe molecules comprising RNA, DNA, or RNA/DNA hybrid sequences of any length in single-stranded or duplex form. The term "nucleotide" is also used herein as a noun to refer to individual nucleotides or varieties of nucleotides, meaning a molecule, or individual unit in a larger nucleic acid molecule, comprising a purine or pyrimidine, a ribose or deoxyribose sugar moiety, and a phosphate group, or phosphodiester linkage in the case of nucleotides within an oligonucleotide or polynucleotide. Although the term "nucleotide" is also used herein to encompass "modified nucleotides" which comprise at least one modifications (a) an alternative linking group, (b) an analogous form of purine, (c) an analogous form of pyrimidine, or (d) an analogous sugar, for examples of analogous linking groups, purine, pyrimidines, and sugars see for example PCT publication No. WO 95/04064. This may be especially oligonucleotides with α or β anomers, oligonucleotides with inter-nucleotide linkage of the phosphorothioate or methyl phosphonate type, or alternatively oligothionucleotide. The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, *ex vivo* generation, or a combination thereof, as well as utilizing any purification methods known in the art.

Throughout the present specification, the expression "nucleotide sequence" may be employed to designate indifferently a polynucleotide or a nucleic acid. More precisely, the expression "nucleotide sequence" encompasses the nucleic material itself and is thus not restricted to the sequence information (i.e. the succession of letters chosen among the four base letters) that biochemically characterizes a specific DNA or RNA molecule.

A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell required to initiate the specific transcription of a gene.

A sequence which is "operably linked" to a regulatory sequence such as a promoter means that said regulatory element is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the nucleic acid of interest. As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. More precisely, two DNA molecules (such as a polynucleotide containing a promoter region and a polynucleotide encoding a desired polypeptide or polynucleotide) are said to be "operably linked" if the nature of the linkage between the two polynucleotides does not (1) result in the introduction of a

frame-shift mutation or (2) interfere with the ability of the polynucleotide containing the promoter to direct the transcription of the coding polynucleotide.

The term "primer" denotes a specific oligonucleotide sequence which is complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, e.g., polynucleotide as defined herein) which can be used to identify a specific polynucleotide sequence present in samples, said nucleic acid segment comprising a nucleotide sequence complementary of the specific polynucleotide sequence to be identified.

The terms "base paired" and "Watson & Crick base paired" are used interchangeably herein to refer to nucleotides which can be hydrogen bonded to one another by virtue of their sequence identities in a manner like that found in double-helical DNA with thymine or uracil residues linked to adenine residues by two hydrogen bonds and cytosine and guanine residues linked by three hydrogen bonds (See Stryer, L., *Biochemistry,* 4th edition, 1995).

The terms "complementary" or "complement thereof" are used herein to refer to the sequences of polynucleotides which are capable of forming Watson & Crick base pairing with another specified polynucleotide throughout the entirety of the complementary region. For the purpose of the present invention, a first polynucleotide is deemed to be complementary to a second polynucleotide when each base in the first polynucleotide is paired with its complementary base. Complementary bases are, generally, A and T (or A and U), or C and G. "Complement" is used herein as a synonym of "complementary polynucleotide", "complementary nucleic acid" and "complementary nucleotide sequence". These terms are applied to pairs of polynucleotides based solely upon their sequences and not any particular set of conditions under which the two polynucleotides would actually bind.

The term "polypeptide" refers to a polymer of amino acids without regard to the length of the polymer; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude post-expression modifications of polypeptides, for example, polypeptides which include the covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like are expressly encompassed by the term polypeptide. Also included within the definition are polypeptides which contain one or more analogs of an amino acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term "recombinant polypeptide" is used herein to refer to polypeptides that have been artificially designed and which comprise at least two polypeptide sequences that are not found as contiguous polypeptide sequences in their initial natural environment, or to refer to polypeptides which have been expressed from a recombinant polynucleotide.

The term "purified" is used herein to describe a polypeptide of the invention which has been separated from other compounds including, but not limited to nucleic acids, lipids, carbohydrates and other proteins. A polypeptide is substantially pure when at least about 50%, preferably 60 to 75% of a sample exhibits a single polypeptide sequence. A substantially pure polypeptide typically comprises about 50%, preferably 60 to 90% weight/weight of a protein sample, more usually about 95%, and preferably is over about 99% pure. Polypeptide purity or homogeneity is indicated by a number of means well known in the art, such as agarose or polyacrylamide gel electrophoresis of a sample, followed by visualizing a single polypeptide band upon staining the gel. For certain purposes higher resolution can be provided by using HPLC or other means well known in the art.

As used herein, the term "non-human animal" refers to any non-human vertebrate, birds and more usually mammals, preferably primates, farm animals such as swine, goats, sheep, donkeys, and horses, rabbits or rodents, more preferably rats or mice. As used herein, the term "animal" is used to refer to any vertebrate, preferable a mammal. Both the terms "animal" and "mammal" expressly embrace human subjects unless preceded with the term "non-human".

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one binding domain, where an antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, which allows an immunological reaction with the antigen. Antibodies include recombinant proteins comprising the binding domains, as wells as fragments, including Fab, Fab', $F(ab)_2$, and $F(ab')_2$ fragments.

As used herein, an "antigenic determinant" is the portion of an antigen molecule, in this case a PCTA-1 polypeptide, that determines the specificity of the antigen-antibody reaction. An "epitope" refers to an antigenic determinant of a polypeptide. An epitope can comprise as few as 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 6 such amino acids, and more usually at least 8-10 such amino acids. Methods for determining the amino acids which make up an epitope include x-ray crystallography, 2-dimensional nuclear magnetic resonance, and epitope mapping e.g. the Pepscan method described by Geysen et al. 1984; PCT Publication No. WO 84/03564; and PCT

Publication No. WO 84/03506, the disclosures of which are incorporated herein by reference in their entireties.

The term "allele" is used herein to refer to variants of a nucleotide sequence. A biallelic polymorphism has two forms. Diploid organisms may be homozygous or heterozygous for an allelic form.

The term "heterozygosity rate" is used herein to refer to the incidence of individuals in a population which are heterozygous at a particular allele. In a biallelic system, the heterozygosity rate is on average equal to $2P_a(1-P_a)$, where P_a is the frequency of the least common allele. In order to be useful in genetic studies, a genetic marker should have an adequate level of heterozygosity to allow a reasonable probability that a randomly selected person will be heterozygous.

The term "genotype" as used herein refers the identity of the alleles present in an individual or a sample. In the context of the present invention, a genotype preferably refers to the description of the biallelic marker alleles present in an individual or a sample. The term "genotyping" a sample or an individual for a biallelic marker consists of determining the specific allele or the specific nucleotide carried by an individual at a biallelic marker.

The term "mutation" as used herein refers to a difference in DNA sequence between or among different genomes or individuals which has a frequency below 1%.

The term "haplotype" refers to a combination of alleles present in an individual or a sample. In the context of the present invention, a haplotype preferably refers to a combination of biallelic marker alleles found in a given individual and which may be associated with a phenotype.

The term "polymorphism" as used herein refers to the occurrence of two or more alternative genomic sequences or alleles between or among different genomes or individuals. "Polymorphic" refers to the condition in which two or more variants of a specific genomic sequence can be found in a population. A "polymorphic site" is the locus at which the variation occurs. A single nucleotide polymorphism is a single base pair change. Typically a single nucleotide polymorphism is the replacement of one nucleotide by another nucleotide at the polymorphic site. Deletion of a single nucleotide or insertion of a single nucleotide, also give rise to single nucleotide polymorphisms. In the context of the present invention "single nucleotide polymorphism" preferably refers to a single nucleotide substitution. However, the polymorphism can also involve an insertion or a deletion of at least one nucleotide, preferably between 1 and 5 nucleotides. The nucleotide modification can also involve the presence of several adjacent single base polymorphisms. This type of nucleotide modification is usually called a "variable motif". Generally, a "variable motif" involves the presence of 2 to 10

adjacent single base polymorphisms. In some instances, series of two or more single base polymorphisms can be interrupted by single bases which are not polymorphic. This is also globally considered to be a "variable motif". Typically, between different genomes or between different individuals, the polymorphic site may be occupied by two different nucleotides.

The term "biallelic polymorphism" and "biallelic marker" are used interchangeably herein to refer to a polymorphism, usually a single nucleotide, having two alleles at a fairly high frequency in the population. A "biallelic marker allele" refers to the nucleotide variants present at a biallelic marker site. Typically, the frequency of the less common allele of the biallelic markers of the present invention has been validated to be greater than 1%, preferably the frequency is greater than 10%, more preferably the frequency is at least 20% (i.e. heterozygosity rate of at least 0.32), even more preferably the frequency is at least 30% (i.e. heterozygosity rate of at least 0.42). A biallelic marker wherein the frequency of the less common allele is 30% or more is termed a "high quality biallelic marker".

As used herein the terminology "defining a biallelic marker" means that a sequence includes a polymorphic base from a biallelic marker. The sequences defining a biallelic marker may be of any length consistent with their intended use, provided that they contain a polymorphic base from a biallelic marker. The sequence has between 2 and 100, preferably between 20, 30, or 40 and 60, and more preferably about 47 nucleotides in length. Likewise, the term "marker" or "biallelic marker" requires that the sequence is of sufficient length to practically (although not necessarily unambiguously) identify the polymorphic allele, which usually implies a length of at least 4, 5, 6, 10, 15, 20, 25, or 40 nucleotides.

As used herein the term "*PCTA-1*-related biallelic marker " or "biallelic marker of the *PCTA-1* gene" relates to a set of biallelic markers in linkage disequilibrium with the *PCTA-1* gene. The term *PCTA-1*-related biallelic marker encompasses all of the biallelic markers A1 to A125 disclosed in Table 2.

The location of nucleotides in a polynucleotide with respect to the center of the polynucleotide are described herein in the following manner. When a polynucleotide has an odd number of nucleotides, the nucleotide at an equal distance from the 3' and 5' ends of the polynucleotide is considered to be "at the center" of the polynucleotide, and any nucleotide immediately adjacent to the nucleotide at the center, or the nucleotide at the center itself is considered to be "within 1 nucleotide of the center." With an odd number of nucleotides in a polynucleotide any of the five nucleotides positions in the middle of the polynucleotide would be considered to be within 2 nucleotides of the center, and so on. When a polynucleotide has an even number of nucleotides, there would be a bond and not a nucleotide at the center of the polynucleotide. Thus, either of the two central nucleotides would be considered to be "within 1

nucleotide of the center" and any of the four nucleotides in the middle of the polynucleotide would be considered to be "within 2 nucleotides of the center", and so on. For polymorphisms which involve the substitution, insertion or deletion of 1 or more nucleotides, the polymorphism, allele or biallelic marker is "at the center" of a polynucleotide if the difference between the distance from the substituted, inserted, or deleted polynucleotides of the polymorphism and the 3' end of the polynucleotide, and the distance from the substituted, inserted, or deleted polynucleotides of the polymorphism and the 5' end of the polynucleotide is zero or one nucleotide. If this difference is 0 to 3, then the polymorphism is considered to be "within 1 nucleotide of the center." If the difference is 0 to 5, the polymorphism is considered to be "within 2 nucleotides of the center." If the difference is 0 to 7, the polymorphism is considered to be "within 3 nucleotides of the center," and so on.

The terms "trait" and "phenotype" are used interchangeably herein and refer to any visible, detectable or otherwise measurable property of an organism such as symptoms of, or susceptibility to a disease for example. Preferably, the term "trait" or "phenotype", when used herein, encompasses, but is not limited to, prostate cancer, an early onset of prostate cancer, a beneficial response to or side effects related to treatment or a vaccination against prostate cancer, a susceptibility to prostate cancer, the level of aggressiveness of prostate cancer tumors, a modified or forthcoming expression of the *PCTA-1* gene, a modified or forthcoming production of the PCTA-1 protein, or the production of a modified PCTA-1 protein. However, the term "trait" or "phenotype" can refer to other types of cancer.

The term "susceptibility to prostate cancer" is used herein to designate a strong likelihood for an individual to develop in his lifetime a form of prostate cancer, particularly a form of prostate cancer in which a PCTA-1 protein is expressed. This likelihood is strongly related to the association established between the biallelic markers of the present invention and prostate cancer or other more specific characteristics which can lead to the development of the prostate cancer such as the modified expression of the *PCTA-1* gene, the modified production of the PCTA-1 protein or the production of a modified PCTA-1 protein.

The term "aggressiveness" of prostate cancer tumors refers to the metastatic potential of these tumors.

The term "treatment of prostate cancer" when used herein is intended to designate the administration of substances either for prophylactic or curative purposes. When administered for prophylactic purposes, the treatment is provided in advance of the appearance of biologically or clinically significant cancer symptoms. When administered for curative purposes, the treatment is provided to attenuate the pathological symptoms of prostate cancer, to decrease the size or growth of cancer tumors or metastases or to remove them.

The terms "an agent acting against prostate cancer" refers to any drug or compound that is capable of reducing the growth rate, rate of metastasis, or viability of tumor cells in a mammal, is capable of reducing the size or eliminating tumors in a mammal, or is capable of increasing the average life span of a mammal or human with cancer. Agents acting against prostate cancer also include compounds which are able to reduce the risk of cancer developing in a population, particularly a high risk population. Examples of agents acting against prostate cancer include hormonal therapeutic agents (for example, medroxyprogesterone acetate, estramustine phosphate, gonadotrophin releasing hormone (GnRH) agonists, anti-androgens such as flutamide, nilutamide, groserelin, and cyprosterone acetate, anti-gonadotropic agents such as stilboestrol and other oestrogenic agents, progestogens such as megestrol acetate) or chemotherapeutic agents (for example, carboplatin, cisplatin, methotrexate, mitomycin, epirubicin, vinblastine, 5-fluorouracyl, mitozantrone, cyclophosphamide, interferon, N-(4-hydroxyphenyl) retinamide (4HPR)). These agents can be used in combination.

The term "side effects to an agent acting against prostate cancer" refers to adverse effects of therapy resulting from extensions of the principal pharmacological action of the drug or to idiosyncratic adverse reactions resulting from an interaction of the drug with unique host factors. These side effects include, but are not limited to, adverse reactions such as dermatological, hematological or hepatological toxicities and further includes gastric and intestinal ulceration, disturbance in platelet function, renal injury, nephritis, vasomotor rhinitis with profuse watery secretions, angioneurotic edema, generalized urticaria, and bronchial asthma to laryngeal edema and bronchoconstriction, hypotension, sexual dysfunction, and shock. More particularly, the side effects can be nausea/vomiting, cardiovascular side effects such as deep vein thrombosis and fluid retention, and gynaecomastia.

The term "response to an agent acting against prostate cancer" refers to drug efficacy, including but not limited to ability to metabolize a compound, to the ability to convert a pro-drug to an active drug, and to the pharmacokinetics (absorption, distribution, elimination) and the pharmacodynamics (receptor-related) of a drug in an individual.

In the context of the present invention, a "positive response" to a medicament can be defined as comprising a reduction of the symptoms related to the disease, an increase of survival time or condition to be treated.

In the context of the present invention, a "negative response" to a medicament can be defined as comprising either a lack of positive response to the medicament which does not lead to a symptom reduction or an increase of survival time, or which leads to a side-effect observed following administration of the medicament.

Variants and fragments

1- Polynucleotides

The invention also relates to variants and fragments of the polynucleotides described herein, particularly of a *PCTA-1* gene containing one or more biallelic markers according to the invention.

Variants of polynucleotides, as the term is used herein, are polynucleotides that differ from a reference polynucleotide. A variant of a polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical.

Variants of polynucleotides according to the invention include, without being limited to, nucleotide sequences which are at least 95% identical to a polynucleotide selected from the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8 or to any polynucleotide fragment of at least 8 consecutive nucleotides of a polynucleotide selected from the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8, and preferably at least 99% identical, more particularly at least 99.5% identical, and most preferably at least 99.8% identical to a polynucleotide selected from the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8 or to any polynucleotide fragment of at least 8 consecutive nucleotides of a polynucleotide selected from the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8.

Nucleotide changes present in a variant polynucleotide may be silent, which means that they do not alter the amino acids encoded by the polynucleotide. However, nucleotide changes may also result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

In the context of the present invention, particularly preferred embodiments are those in which the polynucleotides encode polypeptides which retain substantially the same biological function or activity as the mature PCTA-1 protein, or those in which the polynucleotides encode polypeptides which maintain or increase a particular biological activity, while reducing a second biological activity.

A polynucleotide fragment is a polynucleotide having a sequence that entirely is the same as part but not all of a given nucleotide sequence, preferably the nucleotide sequence of a *PCTA-1* gene, and variants thereof. The fragment can be a portion of an exon or of an intron of a *PCTA-1* gene. It can also be a portion of the regulatory sequences of the *PCTA-1* gene, preferably of the promoter. Preferably, such fragments comprise at least one of the biallelic markers A1 to A125, and the complements thereof, or a biallelic marker in linkage disequilibrium therewith.

Such fragments may be "free-standing", i.e. not part of or fused to other polynucleotides, or they may be comprised within a single larger polynucleotide of which they form a part or region. However, several fragments may be comprised within a single larger polynucleotide.

As representative examples of polynucleotide fragments of the invention, there may be mentioned those which have from about 4, 6, 8, 15, 20, 25, 40, 10 to 30, 30 to 55, 50 to 100, 75 to 100 or 100 to 200 nucleotides in length. Preferred are those fragments having about 47 nucleotides in length, such as those of P1 to P125 and the complementary sequences thereto, and containing at least one of the biallelic markers of the *PCTA-1* gene which are described herein. It will of course be understood that the polynucleotides P1 to P125 and the complementary sequences thereto can be shorter or longer, although it is preferred that they at least contain the biallelic marker of the primer which can be located at one end of the fragment.

2- Polypeptides

The invention also relates to variants, fragments, analogs and derivatives of the polypeptides described herein, including mutated PCTA-1 proteins.

The variant may be 1) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, or 2) one in which one or more of the amino acid residues includes a substituent group, or 3) one in which the PCTA-1 protein is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or 4) one in which the additional amino acids are fused to the PCTA-1 protein, such as a leader or secretory sequence or a sequence which is employed for purification of the PCTA-1 protein or a preprotein sequence. Such variants are deemed to be within the scope of those skilled in the art.

A polypeptide fragment is a polypeptide having a sequence that entirely is the same as part but not all of a given polypeptide sequence, preferably a polypeptide encoded by a *PCTA-1* gene and variants thereof. Preferred fragments include those of the active region of the PCTA-1

protein that may play a role in prostate cancer and those regions possessing antigenic properties and which can be used to raise antibodies against the PCTA-1 protein.

In the case of an amino acid substitution in the amino acid sequence of a polypeptide according to the invention, one or several amino acids can be replaced by "equivalent" amino acids. The expression "equivalent" amino acid is used herein to designate any amino acid that may be substituted for one of the amino acids having similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Generally, the following groups of amino acids represent equivalent changes: (1) Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr; (2) Cys, Ser, Tyr, Thr; (3) Val, Ile, Leu, Met, Ala, Phe; (4) Lys, Arg, His; (5) Phe, Tyr, Trp, His.

A specific embodiment of a modified PCTA-1 peptide molecule of interest according to the present invention, includes, but is not limited to, a peptide molecule which is resistant to proteolysis, is a peptide in which the -CONH- peptide bond is modified and replaced by a (CH_2NH) reduced bond, a (NHCO) retro inverso bond, a (CH_2-O) methylene-oxy bond, a (CH_2-S) thiomethylene bond, a ($CH2CH_2$) carba bond, a (CO-CH_2) cetomethylene bond, a (CHOH-CH_2) hydroxyethylene bond), a (N-N) bound, a E-alcene bond or also a -CH=CH- bond. The invention also encompasses a human PCTA-1 polypeptide or a fragment or a variant thereof in which at least one peptide bound has been modified as described above.

Such fragments may be "free-standing", i.e. not part of or fused to other polypeptides, or they may be comprised within a single larger polypeptide of which they form a part or region. However, several fragments may be comprised within a single larger polypeptide.

As representative examples of polypeptide fragments of the invention, there may be mentioned those which have from about 5, 6, 7, 8, 9 or 10 to 15, 10 to 20, 15 to 40, or 30 to 55 amino acids long. Preferred are those fragments containing at least one amino acid mutation in the PCTA-1 protein.

Identity Between Nucleic Acids Or Polypeptides

The terms "percentage of sequence identity" and "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched

positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Homology is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, 1988; Altschul et al., 1990; Thompson et al., 1994; Higgins et al., 1996; Altschul et al., 1993). In a particularly preferred embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") which is well known in the art (see, e.g., Karlin and Altschul, 1990; Altschul et al., 1990, 1993, 1997). In particular, five specific BLAST programs are used to perform the following task:

(1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;

(2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;

(3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database;

(4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and

(5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet et al., 1992; Henikoff and Henikoff, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978). The BLAST programs evaluate the statistical significance of all high-scoring segment pairs identified, and preferably selects those segments which satisfy a user-specified threshold of significance, such as a user-specified percent homology. Preferably, the statistical significance of a high-scoring segment pair is evaluated using the statistical significance formula of Karlin (see, e.g., Karlin and Altschul, 1990).

Stringent Hybridization Conditions

By way of example and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C, the preferred hybridization temperature, in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Alternatively, the hybridization step can be performed at 65°C in the presence of SSC buffer, 1 x SSC corresponding to 0.15M NaCl and 0.05 M Na citrate. Subsequently, filter washes can be done at 37°C for 1 h in a solution containing 2 x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA, followed by a wash in 0.1 X SSC at 50°C for 45 min. Alternatively, filter washes can be performed in a solution containing 2 x SSC and 0.1% SDS, or 0.5 x SSC and 0.1% SDS, or 0.1 x SSC and 0.1% SDS at 68°C for 15 minute intervals. Following the wash steps, the hybridized probes are detectable by autoradiography. Other conditions of high stringency which may be used are well known in the art and as cited in Sambrook et al., 1989; and Ausubel et al., 1989. These hybridization conditions are suitable for a nucleic acid molecule of about 20 nucleotides in length. There is no need to say that the hybridization conditions described above are to be adapted according to the length of the desired nucleic acid, following techniques well known to the one skilled in the art. The suitable hybridization conditions may for example be adapted according to the teachings disclosed in the book of Hames and Higgins (1985) or in Sambrook et al.(1989).

Genomic Sequence Of The PCTA-1 Gene

The present invention relates to a purified and/or isolated nucleic acid corresponding to the genomic sequence of the *PCTA-1* gene. Preferably, this genomic *PCTA-1* sequence comprises the nucleotide sequence of SEQ ID No 1, a sequence complementary thereto, a fragment or a variant thereof.

The present invention encompasses the genomic sequence of *PCTA-1*. The *PTCA-1* gene sequence comprises a coding sequence including 13 exons included in SEQ ID No 1, namely exon 0, exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 6bis, exon 7, exon 8, exon 9, exon 9bis and exon 9ter, the intronic regions, the promoter, the 5'UTR, the 3'UTR, and regulatory regions located upstream and downstream of the coding region.

The localization of the exons and introns of the *PCTA-1* gene is detailed in Table A and is described as feature in SEQ ID No 1.

Table A

Exon	Position range in SEQ ID No 1		Intron	Position range in SEQ ID No 1	
	Beginning	End		Beginning	End
0	68648	68741	0	68742	70646
1	70647	70794	1	70795	82207
2	82208	82296	2	82297	83612
3	83613	83823	3	83824	85297
4	85298	85417	4	85418	86388
5	86389	86445	5	86446	87495
6	87496	87522	6	87523	87649
6bis	87650	87775	6bis	87776	88294
7	88295	88383	7	88384	89483
8	89484	89649	8	89650	92748
9	92749	97155	9bis	92884	95820
9bis	92749	92883			
9ter	95821	97155			

Intron 0 refers to the nucleotide sequence located between Exon 0 and Exon 1, and so on. The intron 6 refers to the nucleotide sequence located between Exon 6 and Exon 6bis. The intron 6bis refers to the nucleotide sequence located between Exon 6bis and Exon 7. The intron 8 refers to the nucleotide sequence located between Exon 8 and Exon 9 or 9bis. The intron 9bis refers to the nucleotide sequence located between Exon 9bis and Exon 9ter.

The invention also encompasses a purified, isolated, or recombinant polynucleotide comprising a nucleotide sequence having at least 70, 75, 80, 85, 90, or 95% nucleotide identity with a nucleotide sequence of SEQ ID No 1 or a complementary sequence thereto or a fragment thereof. The nucleotide differences as regards to the nucleotide sequence of SEQ ID No 1 may be generally randomly distributed throughout the entire nucleic acid. Nevertheless, preferred nucleic acids are those wherein the nucleotide differences as regards to the nucleotide sequence of SEQ ID No 1 are predominantly located outside the coding sequences contained in the exons. These nucleic acids, as well as their fragments and variants, may be used as oligonucleotide primers or probes in order to detect the presence of a copy of the *PCTA-1* gene in a test sample, or alternatively in order to amplify a target nucleotide sequence within the *PCTA-1* sequences.

Another object of the invention consists of a purified, isolated, or recombinant nucleic acid that hybridizes with the nucleotide sequence of SEQ ID No 1 or a complementary sequence thereto or a variant thereof, under the stringent hybridization conditions as defined above.

Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the

following nucleotide positions of SEQ ID No 1: 1-70715, 70795-82207, 82297-83612, 83824-85297, 85418-86388, 86446-87495, 87523-88294, 88384-89483, 89650-92748, 97156-98309, 98476-99329, 99491-100026, 100212-100281, 100396-100538, 100682-100833, 100995-101920, 102087-102970, 103264-103724, and 103753-106746. Additional preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 70728, 87860, 88297, 94432, and 95340 of SEQ ID No 1; a nucleotide A at positions 82218, 83644, 83808, 87787, 87806, 94218, and 97144 of SEQ ID No 1; a nucleotide C at positions 87902, 88215, 88283, 92760, 93726, and 94422 of SEQ ID No 1; and a nucleotide T at positions 93903, and 94170 of SEQ ID No 1. Other preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 86435, 93592, 93680, 93681, 93682, 93728, 93761, and 95445 of SEQ ID No 1; a nucleotide A at positions 86434, 88355, 93240, 93471, and 93747of SEQ ID No 1; a nucleotide C at positions 93683, 95126, and 95444 of SEQ ID No 1; and a nucleotide T at positions 94154, and 94430 of SEQ ID No 1. Other preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 1: 92975-92977, 93711-93715, 94151-94153, 94240-94243, 94770-94773, 94804-94808, 95121-95122, 95129-95135, 95148-95153, 95154-95159, 95173-95178, 95367-95374, 95410-95413, 95418-95420, 95430-95436, 95533-95535, and 95677-95677. It should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section.

A preferred aspect of the present invention is a purified and/or isolated and/or recombined *PCTA-1* gene or a fragment thereof comprising at least one of the biallelic polymorphisms described below, a sequence complementary thereto, a fragment or a variant thereof. In some embodiments, the *PCTA-1* gene or a fragment thereof may comprise at least one of the nucleotide sequences of P1 to P125, a sequence complementary thereto, a fragment or a variant thereof. In a preferred embodiment, the *PCTA-1* gene or a fragment thereof

comprises a biallelic marker selected from the group consisting of A1 to A125 and the complements thereof.

While this section is entitled "Genomic Sequences of The *PCTA-1* Gene", it should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section, flanking the genomic sequences of *PCTA-1* on either side or between two or more such genomic sequences.

PCTA-1 cDNA Sequences

The invention also concerns a purified and/or isolated cDNA encoding a PCTA-1 protein. Preferably, the cDNA comprises a nucleotide sequence selected from the group consisting of SEQ ID Nos 2, 3, 4, sequences complementary thereto and functional fragments and variants thereof. Moreover, preferred polynucleotides of the invention include purified, isolated, or recombinant *PCTA-1* cDNAs consisting of, consisting essentially of, or comprising a sequence selected from the group consisting of SEQ ID Nos 2, 3, 4 and the complementary sequence thereto.

The invention also pertains to a purified or isolated nucleic acid comprising a polynucleotide having at least 95% nucleotide identity with a polynucleotide selected from the group consisting of SEQ ID Nos 2, 3, 4, advantageously 99 % nucleotide identity, preferably 99.5% nucleotide identity and most preferably 99.8% nucleotide identity with a polynucleotide selected from the group consisting of SEQ ID Nos 2, 3, 4, or a sequence complementary thereto or a biologically active fragment thereof.

Another object of the invention consists of purified, isolated or recombinant nucleic acids comprising a polynucleotide that hybridizes, under the stringent hybridization conditions defined herein, with a polynucleotide selected from the group consisting of the nucleotide sequences of SEQ ID Nos 2, 3, 4, or a sequence complementary thereto or a variant thereof or a biologically active fragment thereof.

The 5'UTR and 3'UTR regions of a gene are of particular importance in that they often comprise regulatory elements which can play a role in providing appropriate expression levels, particularly through the control of mRNA stability. The inventors have cloned a complete *PCTA-1* cDNA (SEQ ID No 2) in which the 5'UTR is carried by exon 0 and a portion of exon 1 and the 3'UTR is carried by a portion of exon 9. Moreover, they have characterized a 5'EST, which is located as a feature in SEQ ID No 1, comprising the exons 0 and 1, and partially exon 2. Since an ATG codon is located at the beginning of the partial exon 1 disclosed in WO 96/21671, one could assume that the promoter of the *PCTA-1* gene would be located immediately upstream of this codon. However, the inventors unexpectedly found that the

PCTA-1 genomic DNA contains further exonic sequences upstream of the partial exon 1 disclosed in WO 96/21671. Without the knowledge of such sequences, the identification by the skilled person of the *PCTA-1* promoter was extremely unlikely. Only the full genomic sequence of *PCTA-1* and access by the inventors to a proprietary 5'EST database rendered possible the identification of a full cDNA sequence and of the *PCTA-1* promoter. The invention concern the nucleotide sequence of 5' EST consisting of the position range 1-266 in the SEQ ID No 2.

The main characteristics of the *PCTA-1* cDNA comprising exons 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 are detailed in Table B. The invention concerns the purified and/or isolated sequence of the 5'UTR and 3'UTR as described in Table B or a complementary sequence thereto or an allelic variant thereof set forth in SEQ ID No 2. Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 2. Further preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2471, and 5397 of SEQ ID No 2; a nucleotide C at positions 1013, 1979, and 2675 of SEQ ID No 2; a nucleotide G at positions 176, 749, 2685, 3593 of SEQ ID No 2; and a nucleotide T at positions 2156, and 2423 of SEQ ID No 2. Additional preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1493, 1724, and 2000; a nucleotide C at positions 1936, 3379, and 3697; a nucleotide G at positions 709, 1845, 1933, 1934, 1935, 1981, 2014, and 3698; and a nucleotide T at positions 2407, and 2683 of SEQ ID No 2. Other preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 2: 1229-1231, 1964-1968, 2404-2406, 2493-2496, 3023-3026, 3057-3061, 3374-3375, 3382-3388, 3401-3406, 3407-3412, 3426-3431,

3620-3627, 3663-3666, 3671-3673, 3683-3689, 3786-3788 and 3930-3932. It should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section.

The majority of interrupted genes are transcribed into an RNA that gives rise to a single type of spliced mRNA. But the RNAs of some genes follow patterns of alternative splicing, wherein a single gene gives rise to more than one mRNA species. In some cases, the ultimate pattern of expression is dictated by the primary transcript, because the use of different startpoints or termination sequences alters the splicing pattern. In other cases, a single primary transcript is spliced in more than one way, and internal exons are substituted, added or deleted. In some cases, the multiple products all are made in the same cell, but in others, the process is regulated so that particular splicing patterns occur only under particular conditions.

At least three *PCTA-1* cDNAs are produced by alternative splicing. The inventors have identified a minor species of *PCTA-1* cDNA, disclosed in SEQ ID No 3, and comprising an additional exon 6bis which encodes 42 additional amino acids. In a further embodiment, the present invention concerns the additional exon of the *PCTA-1* gene located between exon 6 and exon 7, namely exon 6bis, detailed as a feature in SEQ ID No 1 and in Table A, a sequence complementary thereto, and a fragment or variant thereof. The present invention embodies a PCTA-1 cDNA comprising the exon 6bis disclosed in SEQ ID No 1.

The main characteristics of this second *PCTA-1* cDNA comprising exons 0, 1, 2, 3, 4, 5, 6, 6bis, 7, 8, and 9 are detailed in Table B. The amino acid sequence of this new PCTA-1 protein is disclosed in SEQ ID No 6. Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 3: 1-162 and 747-872. Further preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2597, and 5523 of SEQ ID No 3; a nucleotide C at positions 1139, 2105, and 2801 of SEQ ID No 3; a nucleotide G at positions 176, 875, 2811, 3719 of SEQ ID No 3; and a nucleotide T at positions 2282, and 2549 of SEQ ID No 3. Additional preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the

complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1619, 1850, and 2126; a nucleotide C at positions 2062, 3505, and 3823; a nucleotide G at positions 709, 1971, 2059, 2060, 2061, 2107, 2140, and 3824; and a nucleotide T at positions 2533, and 2809 of SEQ ID No 3. Other preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 3: 1355-1357, 1892-1894, 2090-2094, 2530-2532, 2619-2622, 3149-3152, 3183-3187, 3500-3501, 3508-3514, 3527-3532, 3533-3538, 3552-3557, 3746-3749, 3789-3792, 3797-3799, 3809-3815, 3912-3914 and 4056-4058. It should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section.

The inventors have also identified a species of *PCTA-1* cDNA comprising alternative exons to exon 9 which are called exons 9bis and 9ter. Its sequence is disclosed in SEQ ID No 4. The exon 9bis and 9ter correspond respectively to the beginning and the ends of the exon 9. The polynucleotide of the exon 9 located between exons 9bis and 9ter is spliced or deleted. The combination of exons 9bis and 9ter extends the ORF of the *PCTA-1* gene.

The main characteristics of this second *PCTA-1* cDNA comprising exons 0, 1, 2, 3, 4, 5, 6, 7, 8, 9bis and 9ter are detailed in Table B. The amino acid sequence of the new PCTA-1 protein encoded by this cDNA is disclosed in SEQ ID No 7. Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 4. Further preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527 and 2460 of SEQ ID No 4; a nucleotide C at position 1013 of SEQ ID No 4 and a nucleotide G at positions 176, and 749 of SEQ ID No 4. Additionally preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected

from the group consisting of a nucleotide A at positions 708 and 807 and a nucleotide G at position 709 of SEQ No 4. Other preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises the pairs of nucleotide positions 1136-1137 of SEQ ID No 4. It should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section.

The invention further embodies purified, isolated, or recombinant polynucleotides comprising a nucleotide sequence selected from the group consisting of the 13 exons of the *PCTA-1* gene, or a sequence complementary thereto. The invention also deals with purified, isolated, or recombinant nucleic acids comprising a combination of at least two exons of the *PCTA-1* gene, wherein the polynucleotides are arranged within the nucleic acid, from the 5'-end to the 3'-end of said nucleic acid, in the same order as in SEQ ID No 1. In this specific embodiment of a purified or isolated nucleic acid according to the invention, said nucleic acid preferably comprises the exon 0 at its 5' end and the exon 9 or 9ter at its 3' end.

The 3'UTR sequence of *PCTA*-1 appears to include several polyadenylation sites. These polyadenylation sites could have an influence on the stability of the mRNA resulting from the transcription of the *PCTA-1* genomic DNA.

The invention also concerns a purified and/or isolated cDNA sequence encoding a mouse PCTA-1 protein, particularly a cDNA comprising the nucleotide sequence of SEQ ID No 8, a sequence complementary thereto or a fragment and variant thereof. The main characteristics of the murine cDNA are detailed in Table B. Moreover, preferred polynucleotides of the invention include purified, isolated, or recombinant *PCTA-1* cDNAs consisting of, consisting essentially of, or comprising the sequence of SEQ ID No 8 and the complementary sequence thereto.

The invention also pertains to a purified or isolated nucleic acid comprising a polynucleotide having at least 95% nucleotide identity with a polynucleotide of SEQ ID No 8, advantageously 99 % nucleotide identity, preferably 99.5% nucleotide identity and most preferably 99.8% nucleotide identity with a polynucleotide of SEQ ID No 8, or a sequence complementary thereto or a biologically active fragment thereof.

Another object of the invention consists of purified, isolated or recombinant nucleic acids comprising a polynucleotide that hybridizes, under the stringent hybridization conditions defined herein, with a polynucleotide of SEQ ID No 8, or a sequence complementary thereto or a variant thereof or a biologically active fragment thereof.

Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 8 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 8: 1-500, 501-1000, 1001-1500, and 1501-1738.

Table B

cDNA	Position range of 5'UTR	Position range of ORF		Position range of 3'UTR	Position range of polyadenylation sites
		ATG	STOP		
SEQ ID No 2	1-200	201-203	1149-1151	1152-5408	1773-1778, 3624-3629, 3828-3833, 5119-5124, 5381-5386, 5386-5391
SEQ ID No 3	1-200	201-203	1275-1277	1278-5534	1899-1904, 3750-3755, 3954-3959, 5245-5250, 5507-5512, 5512-5517
SEQ ID No 4	1-200	201-203	1305-1307	1308-2471	2182-2187, 2444-2449, 2449-2454
SEQ ID No 8	1-120	121-123	1068-1070	1071-1738	

While this section is entitled "*PCTA-1* cDNA Sequences," it should be noted that nucleic acid fragments of any size and sequence may also be comprised by the polynucleotides described in this section, flanking the genomic sequences of *PCTA-1* on either side or between two or more such genomic sequences.

Coding Regions

The invention also concerns a nucleotide sequence encoding the human PCTA-1 protein selected from the group consisting of SEQ ID No 5, 6, 7, sequences complementary thereto and fragments and variants thereof. The present invention embodies isolated, purified, and recombinant polynucleotides which encode polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 or 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5.

The present invention also embodies isolated, purified, and recombinant polynucleotides which encode polypeptides comprising a contiguous span of at least 6 amino

acids, preferably at least 8 or 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

- at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6.

The present invention further embodies isolated, purified, and recombinant polynucleotides which encode polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 or 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

- at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7.

The invention also concerns a nucleotide sequence encoding the murine PCTA-1 protein of SEQ ID No 9, sequences complementary thereto and fragments and variants thereof. More particularly, the present invention embodies isolated, purified, and recombinant polynucleotides which encode polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 or 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following amino acid positions of SEQ ID No 9: 1-50, 51-100, 101-150, 151-200, 201-250, and 251-316.

The above disclosed polynucleotide that contains the coding sequence of the *PCTA-1* gene may be expressed in a desired host cell or a desired host organism, when this polynucleotide is placed under the control of suitable expression signals. The expression signals may be either the expression signals contained in the regulatory regions in the *PCTA-1* gene of the invention or in contrast the signals may be exogenous regulatory nucleic sequences. Such a polynucleotide, when placed under the suitable expression signals, may also be inserted in a vector for its expression and/or amplification.

Regulatory Sequences Of The *PCTA-1* Gene

The present invention also concerns the purified and/or isolated sequences of the upstream regulatory region (5' regulatory region) of the *PCTA-1* gene, sequences complementary thereto, and fragments or variants thereof, particularly the nucleotide sequence located between positions 1 and 68647 of SEQ ID No 1, as well as any sequence of 8 to 3000 consecutive nucleotides, preferably of 8 to 500 consecutive nucleotides, included therein. More particularly, the invention further includes specific elements within this regulatory region. These elements include a promoter region. The promoter region appears to be located in the 10 kb region, preferably in the 5 kb region, more preferably in the 2 kb region, still more preferably in the 1 kb region, and more particularly in the 500 bp, upstream of the first exon of the *PCTA-1* gene. Preferably, the promoter region has a nucleotide sequence located between positions 66647 and 68647 of SEQ ID No 1 as well as any functional sequence of at least 8 consecutive nucleotide, preferably 8 to 400 consecutive nucleotides, more preferably of 8 to 300 nucleotides included therein, sequences complementary thereto and fragments and variants thereof. Further comments are provided below on this region which is of a particular importance in the present invention.

Also included in the invention are regulatory sequences downstream of the *PCTA-1* coding sequence (3' regulatory region) such as those included in the nucleotide sequence located between positions 97156 and 106746 of SEQ ID No 1, sequences complementary thereto and fragments and variants thereof.

In order to identify the relevant biologically active polynucleotide fragments or variants of the 5' or 3' regulatory region, the one skilled in the art will refer to the book of Sambrook et al. (Sambrook et al., 1989) which describes the use of a recombinant vector carrying a marker gene (i.e. beta galactosidase, chloramphenicol acetyl transferase, etc.) the expression of which will be detected when placed under the control of a biologically active polynucleotide fragments or variants of the 5' or 3' regulatory region. Genomic sequences located upstream of the first exon of the *PCTA-1* gene are cloned into a suitable promoter reporter vector, such as the pSEAP-Basic, pSEAP-Enhancer, pβgal-Basic, pβgal-Enhancer, or pEGFP-1 Promoter Reporter vectors available from Clontech, or pGL2-basic or pGL3-basic promoterless luciferase reporter gene vector from Promega. Briefly, each of these promoter reporter vectors include multiple cloning sites positioned upstream of a reporter gene encoding a readily assayable protein such as secreted alkaline phosphatase, luciferase, beta galactosidase, or green fluorescent protein. The sequences upstream the *PCTA-1* coding region are inserted into the cloning sites upstream of the reporter gene in both orientations and introduced into an appropriate host cell. The level of reporter protein is assayed and compared to the level obtained from a vector which lacks an

insert in the cloning site. The presence of an elevated expression level in the vector containing the insert with respect to the control vector indicates the presence of a promoter in the insert. If necessary, the upstream sequences can be cloned into vectors which contain an enhancer for increasing transcription levels from weak promoter sequences. A significant level of expression above that observed with the vector lacking an insert indicates that a promoter sequence is present in the inserted upstream sequence.

Promoter sequences within the upstream genomic DNA may be further defined by constructing nested 5' and/or 3' deletions in the upstream DNA using conventional techniques such as Exonuclease III or appropriate restriction endonuclease digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity. In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into cloning sites in promoter reporter vectors. This type of assay is well-known to those skilled in the art and is described in WO 97/17359, US 5,374,544, EP 582,796, US 5,698,389, US 5,643,746, US 5,502,176, and US 5,266,488, the disclosures of which are incorporated herein by reference in their entireties.

The strength and the specificity of the promoter of the *PCTA-1* gene can be assessed through the expression levels of a detectable polynucleotide operably linked to the *PCTA-1* promoter in different types of cells and tissues. The detectable polynucleotide may be either a polynucleotide that specifically hybridizes with a predefined oligonucleotide probe, or a polynucleotide encoding a detectable protein, including a PCTA-1 polypeptide or a fragment or a variant thereof. This type of assay is well-known to those skilled in the art and is described in US 5,502,176, and US 5,266,488, the disclosures of which are incorporated herein by reference in their entireties. In one embodiment, the efficacy of the promoter of the *PCTA-1* gene is assessed in normal and cancer cells. In a preferred embodiment, the efficacy of the promoter of the *PCTA-1* gene is assessed in normal cells and in cancer cells which can present different degrees of malignancy, more preferably cells from prostate tissue. Some of the methods are discussed in more detail below.

Polynucleotides carrying the regulatory elements located at the 5' end and at the 3' end of the *PCTA-1* coding region may be advantageously used to control the transcriptional and translational activity of an heterologous polynucleotide of interest.

Thus, the present invention also concerns a purified or isolated nucleic acid comprising a polynucleotide which is selected from the group consisting of the 5' and 3' regulatory regions, or a sequence complementary thereto or a biologically active fragment or variant thereof. "5' regulatory region" refers to the nucleotide sequence located between positions 1 and 68647 of SEQ ID No 1. "3' regulatory region" refers to the nucleotide sequence located between positions 97156 and 106746 of SEQ ID No 1.

The invention also pertains to a purified or isolated nucleic acid comprising a polynucleotide having at least 95% nucleotide identity with a polynucleotide selected from the group consisting of the 5' and 3' regulatory regions, advantageously 99 % nucleotide identity, preferably 99.5% nucleotide identity and most preferably 99.8% nucleotide identity with a polynucleotide selected from the group consisting of the 5' and 3' regulatory regions, or a sequence complementary thereto or a biologically active fragment thereof.

Another object of the invention consists of purified, isolated or recombinant nucleic acids comprising a polynucleotide that hybridizes, under the stringent hybridization conditions defined herein, with a polynucleotide selected from the group consisting of the nucleotide sequences of the 5'- and 3' regulatory regions, or a sequence complementary thereto or a variant thereof or a biologically active fragment thereof.

Preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 1: 1-4000, 4001-8000, 8001-12000, 12001-16000, 16001-20000, 20001-24000, 24001-28000, 28001-32000, 32001-36000, 36001-40000, 40001-44000, 44001-48000, 48001-52000, 52001-56000, 56001-60000, 60001-64000, 64001-68647. Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 1: 66647-68647.

"Biologically active" polynucleotide derivatives of SEQ ID No 1 are polynucleotides comprising or alternatively consisting of a fragment of said polynucleotide which is functional as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide in a recombinant cell host. It could act either as an enhancer or as a repressor.

For the purpose of the invention, a nucleic acid or polynucleotide is "functional" as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide if

said regulatory polynucleotide contains nucleotide sequences which contain transcriptional and translational regulatory information, and such sequences are "operably linked" to nucleotide sequences which encode the desired polypeptide or the desired polynucleotide.

The regulatory polynucleotides of the invention may be prepared from the nucleotide sequence of SEQ ID No 1 by cleavage using suitable restriction enzymes, as described for example in the book of Sambrook et al.(1989). The regulatory polynucleotides may also be prepared by digestion of SEQ ID No 1 by an exonuclease enzyme, such as Bal31 (Wabiko et al., 1986). These regulatory polynucleotides can also be prepared by nucleic acid chemical synthesis, as described elsewhere in the specification.

A preferred 5'-regulatory polynucleotide of the invention includes the 5'-untranslated region (5'-UTR) of the *PCTA-1* cDNA, or a biologically active fragment or variant thereof. A preferred 3'-regulatory polynucleotide of the invention includes the 3'-untranslated region (3'-UTR) of the *PCTA-1* cDNA, or a biologically active fragment or variant thereof.

A further object of the invention consists of a purified or isolated nucleic acid comprising:

a) a nucleic acid comprising a regulatory nucleotide sequence selected from the group consisting of:

(i) a nucleotide sequence comprising a polynucleotide of the 5' regulatory region or a complementary sequence thereto;

(ii) a nucleotide sequence comprising a polynucleotide having at least 95% of nucleotide identity with the nucleotide sequence of the 5' regulatory region or a complementary sequence thereto;

(iii) a nucleotide sequence comprising a polynucleotide that hybridizes under stringent hybridization conditions with the nucleotide sequence of the 5' regulatory region or a complementary sequence thereto; and

(iv) a biologically active fragment or variant of the polynucleotides in (i), (ii) and (iii);

b) a polynucleotide encoding a desired polypeptide or a nucleic acid of interest, operably linked to the nucleic acid defined in (a) above; and

c) Optionally, a nucleic acid comprising a 3'- regulatory polynucleotide, preferably a 3'- regulatory polynucleotide of the *PCTA-1* gene.

In a specific embodiment of the nucleic acid defined above, said nucleic acid includes the 5'-untranslated region (5'-UTR) of the *PCTA-1* cDNA, or a biologically active fragment or variant thereof. In a second specific embodiment of the nucleic acid defined above, said nucleic

acid includes the 3'-untranslated region (3'-UTR) of the *PCTA-1* cDNA, or a biologically active fragment or variant thereof.

The regulatory polynucleotide of the 5' regulatory region, or its biologically active fragments or variants, is operably linked at the 5'-end of the polynucleotide encoding the desired polypeptide or polynucleotide.

The regulatory polynucleotide of the 3' regulatory region, or its biologically active fragments or variants, is advantageously operably linked at the 3'-end of the polynucleotide encoding the desired polypeptide or polynucleotide.

The desired polypeptide encoded by the above-described nucleic acid may be of various nature or origin, encompassing proteins of prokaryotic or eukaryotic origin. Among the polypeptides expressed under the control of a *PCTA-1* regulatory region include bacterial, fungal or viral antigens. Also encompassed are eukaryotic proteins such as intracellular proteins, like "house keeping" proteins, membrane-bound proteins, like receptors, and secreted proteins like endogenous mediators such as cytokines. The desired polypeptide may be the PCTA-1 protein, especially the protein of a amino acid sequence selected from the group consisting of SEQ ID Nos 5, 6, 7, 9, or a fragment or a variant thereof.

The desired nucleic acids encoded by the above-described polynucleotide, usually an RNA molecule, may be complementary to a desired coding polynucleotide, for example to a *PCTA-1* coding sequence, and thus useful as an antisense polynucleotide.

Such a polynucleotide may be included in a recombinant expression vector in order to express the desired polypeptide or the desired nucleic acid in host cell or in a host organism. Suitable recombinant vectors that contain a polynucleotide such as described herein are disclosed elsewhere in the specification.

Polynucleotide Constructs

The terms "polynucleotide construct" and "recombinant polynucleotide" are used interchangeably herein to refer to linear or circular, purified or isolated polynucleotides that have been artificially designed and which comprise at least two nucleotide sequences that are not found as contiguous nucleotide sequences in their initial natural environment.

DNA Construct That Enables Directing Temporal And Spatial *PCTA-1* Gene Expression In Recombinant Cell Hosts And In Transgenic Animals.

In order to study the physiological and phenotypic consequences of a lack of synthesis of the PCTA-1 protein, both at the cell level and at the multi cellular organism level, the invention also encompasses DNA constructs and recombinant vectors enabling a conditional expression of a specific allele of the *PCTA-1* genomic sequence or cDNA and also of a copy of

this genomic sequence or cDNA harboring substitutions, deletions, or additions of one or more bases as regards to the *PCTA-1* nucleotide sequence of SEQ ID Nos 1, 2, 3, 4, 8, or a fragment thereof, these base substitutions, deletions or additions being located either in an exon, an intron or a regulatory sequence, but preferably in the 5'-regulatory sequence or in an exon of the *PCTA-1* genomic sequence or within a *PCTA-1* cDNA of SEQ ID Nos 2, 3, 4, or 8. In a preferred embodiment, the *PCTA-1* sequence comprises a biallelic marker of the present invention. In a preferred embodiment, the *PCTA-1* sequence comprises a biallelic marker of the present invention, preferably one of the biallelic markers A1 to A125 and the complements thereof.

The present invention embodies recombinant vectors comprising any one of the polynucleotides described in the present invention. More particularly, the polynucleotide constructs according to the present invention can comprise any of the polynucleotides described in the "*PCTA-1* cDNA Sequences" section, the "Coding Regions" section, and the "Oligonucleotide Probes And Primers" section.

A first preferred DNA construct is based on the tetracycline resistance operon *tet* from *E. coli* transposon Tn10 for controlling the *PCTA-1* gene expression, such as described by Gossen et al.(1992, 1995) and Furth et al.(1994). Such a DNA construct contains seven *tet* operator sequences from Tn10 (*tet*op) that are fused to either a minimal promoter or a 5'-regulatory sequence of the *PCTA-1* gene, said minimal promoter or said *PCTA-1* regulatory sequence being operably linked to a polynucleotide of interest that codes either for a sense or an antisense oligonucleotide or for a polypeptide, including a *PCTA-1* polypeptide or a peptide fragment thereof. This DNA construct is functional as a conditional expression system for the nucleotide sequence of interest when the same cell also comprises a nucleotide sequence coding for either the wild type (tTA) or the mutant (rTA) repressor fused to the activating domain of viral protein VP16 of herpes simplex virus, placed under the control of a promoter, such as the HCMVIE1 enhancer/promoter or the MMTV-LTR. Indeed, a preferred DNA construct of the invention comprise both the polynucleotide containing the *tet* operator sequences and the polynucleotide containing a sequence coding for the tTA or the rTA repressor.

In a specific embodiment, the conditional expression DNA construct contains the sequence encoding the mutant tetracycline repressor rTA, the expression of the polynucleotide of interest is silent in the absence of tetracycline and induced in its presence.

DNA Constructs Allowing Homologous Recombination: Replacement Vectors

A second preferred DNA construct will comprise, from 5'-end to 3'-end: (a) a first nucleotide sequence that is comprised in the *PCTA-1* genomic sequence; (b) a nucleotide

sequence comprising a positive selection marker, such as the marker for neomycine resistance (*neo*); and (c) a second nucleotide sequence that is comprised in the *PCTA-1* genomic sequence, and is located on the genome downstream the first *PCTA-1* nucleotide sequence (a).

In a preferred embodiment, this DNA construct also comprises a negative selection marker located upstream the nucleotide sequence (a) or downstream the nucleotide sequence (c). Preferably, the negative selection marker consists of the thymidine kinase (*tk*) gene (Thomas et al., 1986), the hygromycine beta gene (Te Riele et al., 1990), the *hprt* gene (Van der Lugt et al., 1991; Reid et al., 1990) or the Diphteria toxin A fragment (*Dt-A*) gene (Nada et al., 1993; Yagi et al.1990). Preferably, the positive selection marker is located within a *PCTA-1* exon sequence so as to interrupt the sequence encoding a PCTA-1 protein. These replacement vectors are described, for example, by Thomas et al.(1986; 1987), Mansour et al.(1988) and Koller et al.(1992).

The first and second nucleotide sequences (a) and (c) may be indifferently located within a *PCTA-1* regulatory sequence, an intronic sequence, an exon sequence or a sequence containing both regulatory and/or intronic and/or exon sequences. The size of the nucleotide sequences (a) and (c) ranges from 1 to 50 kb, preferably from 1 to 10 kb, more preferably from 2 to 6 kb and most preferably from 2 to 4 kb.

DNA Constructs Allowing Homologous Recombination: Cre-LoxP System.

These new DNA constructs make use of the site specific recombination system of the P1 phage. The P1 phage possesses a recombinase called Cre which interacts specifically with a 34 base pairs *lox*P site. The *lox*P site is composed of two palindromic sequences of 13 bp separated by a 8 bp conserved sequence (Hoess et al., 1986). The recombination by the Cre enzyme between two *lox*P sites having an identical orientation leads to the deletion of the DNA fragment.

The Cre-*lox*P system used in combination with a homologous recombination technique has been first described by Gu et al.(1993, 1994). Briefly, a nucleotide sequence of interest to be inserted in a targeted location of the genome harbors at least two *lox*P sites in the same orientation and located at the respective ends of a nucleotide sequence to be excised from the recombinant genome. The excision event requires the presence of the recombinase (Cre) enzyme within the nucleus of the recombinant cell host. The recombinase enzyme may be brought at the desired time either by (a) incubating the recombinant cell hosts in a culture medium containing this enzyme, by injecting the Cre enzyme directly into the desired cell, such as described by Araki et al.(1995), or by lipofection of the enzyme into the cells, such as described by Baubonis et al.(1993); (b) transfecting the cell host with a vector comprising the

Cre coding sequence operably linked to a promoter functional in the recombinant cell host, which promoter being optionally inducible, said vector being introduced in the recombinant cell host, such as described by Gu et al.(1993) and Sauer et al.(1988); (c) introducing in the genome of the cell host a polynucleotide comprising the *Cre* coding sequence operably linked to a promoter functional in the recombinant cell host, which promoter is optionally inducible, and said polynucleotide being inserted in the genome of the cell host either by a random insertion event or an homologous recombination event, such as described by Gu et al.(1994).

In a specific embodiment, the vector containing the sequence to be inserted in the *PCTA-1* gene by homologous recombination is constructed in such a way that selectable markers are flanked by *lox*P sites of the same orientation, it is possible, by treatment by the Cre enzyme, to eliminate the selectable markers while leaving the *PCTA-1* sequences of interest that have been inserted by an homologous recombination event. Again, two selectable markers are needed: a positive selection marker to select for the recombination event and a negative selection marker to select for the homologous recombination event. Vectors and methods using the Cre-*lox*P system are described by Zou et al.(1994).

Thus, a third preferred DNA construct of the invention comprises, from 5'-end to 3'-end: (a) a first nucleotide sequence that is comprised in the *PCTA-1* genomic sequence; (b) a nucleotide sequence comprising a polynucleotide encoding a positive selection marker, said nucleotide sequence comprising additionally two sequences defining a site recognized by a recombinase, such as a *lox*P site, the two sites being placed in the same orientation; and (c) a second nucleotide sequence that is comprised in the *PCTA-1* genomic sequence, and is located on the genome downstream of the first *PCTA-1* nucleotide sequence (a).

The sequences defining a site recognized by a recombinase, such as a *lox*P site, are preferably located within the nucleotide sequence (b) at suitable locations bordering the nucleotide sequence for which the conditional excision is sought. In one specific embodiment, two *lox*P sites are located at each side of the positive selection marker sequence, in order to allow its excision at a desired time after the occurrence of the homologous recombination event.

In a preferred embodiment of a method using the third DNA construct described above, the excision of the polynucleotide fragment bordered by the two sites recognized by a recombinase, preferably two loxP sites, is performed at a desired time, due to the presence within the genome of the recombinant host cell of a sequence encoding the Cre enzyme operably linked to a promoter sequence, preferably an inducible promoter, more preferably a tissue-specific promoter sequence and most preferably a promoter sequence which is both inducible and tissue-specific, such as described by Gu et al.(1994).

The presence of the Cre enzyme within the genome of the recombinant cell host may result of the breeding of two transgenic animals, the first transgenic animal bearing the *PCTA-1*-derived sequence of interest containing the *lox*P sites as described above and the second transgenic animal bearing the *Cre* coding sequence operably linked to a suitable promoter sequence, such as described by Gu et al.(1994).

Spatio-temporal control of the Cre enzyme expression may also be achieved with an adenovirus based vector that contains the Cre gene thus allowing infection of cells, or *in vivo* infection of organs, for delivery of the Cre enzyme, such as described by Anton and Graham (1995) and Kanegae et al.(1995).

The DNA constructs described above may be used to introduce a desired nucleotide sequence of the invention, preferably a *PCTA-1* genomic sequence or a *PCTA-1* cDNA sequence, and most preferably an altered copy of a *PCTA-1* genomic or cDNA sequence, within a predetermined location of the targeted genome, leading either to the generation of an altered copy of a targeted gene (knock-out homologous recombination) or to the replacement of a copy of the targeted gene by another copy sufficiently homologous to allow an homologous recombination event to occur (knock-in homologous recombination). In a specific embodiment, the DNA constructs described above may be used to introduce a *PCTA-1* genomic sequence or a *PCTA-1* cDNA sequence comprising at least one biallelic marker of the present invention, preferably at least one biallelic marker selected from the group consisting of A1 to A125 and the complements thereof.

Oligonucleotide Probes And Primers

Polynucleotides derived from the *PCTA-1* gene are useful in order to detect the presence of at least a copy of a nucleotide sequence of SEQ ID No 1, or a fragment, complement, or variant thereof in a test sample.

Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 1: 1-70715, 70795-82207, 82297-83612, 83824-85297, 85418-86388, 86446-87495, 87523-88294, 88384-89483, 89650-92748, 97156-98309, 98476-99329, 99491-100026, 100212-100281, 100396-100538, 100682-100833, 100995-101920, 102087-102970, 103264-103724, and 103753-106746. Additional preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100,

150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 70728, 87860, 88297, 94432, and 95340 of SEQ ID No 1; a nucleotide A at positions 82218, 83644, 83808, 87787, 87806, 94218, and 97144 of SEQ ID No 1; a nucleotide C at positions 87902, 88215, 88283, 92760, 93726, and 94422 of SEQ ID No 1; and a nucleotide T at positions 93903, and 94170 of SEQ ID No 1. Other preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 86435, 93592, 93680, 93681, 93682, 93728, 93761, and 95445 of SEQ ID No 1; a nucleotide A at positions 86434, 88355, 93240, 93471, and 93747of SEQ ID No 1; a nucleotide C at positions 93683, 95126, and 95444 of SEQ ID No 1; and a nucleotide T at positions 94154, and 94430 of SEQ ID No 1. Other preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 1: 92975-92977, 93711-93715, 94151-94153, 94240-94243, 94770-94773, 94804-94808, 95121-95122, 95129-95135, 95148-95153, 95154-95159, 95173-95178, 95367-95374, 95410-95413, 95418-95420, 95430-95436, 95533-95535, and 95677-95677.

Another object of the invention is a purified, isolated, or recombinant polynucleotide comprising the nucleotide sequence of SEQ ID No 2, complementary sequences thereto, as well as allelic variants, and fragments thereof. Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 2. Additional preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2471, and 5397 of SEQ ID No 2; a nucleotide C at positions 1013, 1979, and 2675 of SEQ ID No 2; a nucleotide G at positions 176, 749, 2685, 3593 of SEQ ID No 2; and a nucleotide T at positions 2156, and 2423

of SEQ ID No 2. Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1493, 1724, and 2000; a nucleotide C at positions 1936, 3379, and 3697; a nucleotide G at positions 709, 1845, 1933, 1934, 1935, 1981, 2014, and 3698; and a nucleotide T at positions 2407, and 2683 of SEQ ID No 2. Other preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 2: 1229-1231, 1964-1968, 2404-2406, 2493-2496, 3023-3026, 3057-3061, 3374-3375, 3382-3388, 3401-3406, 3407-3412, 3426-3431, 3620-3627, 3663-3666, 3671-3673, 3683-3689, 3786-3788 and 3930-3932.

A further object of the invention is a purified, isolated, or recombinant polynucleotide comprising the nucleotide sequence of SEQ ID No 3, complementary sequences thereto, as well as allelic variants, and fragments thereof. Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 3: 1-162 and 747-872. Additional preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2597, and 5523 of SEQ ID No 3; a nucleotide C at positions 1139, 2105, and 2801 of SEQ ID No 3; a nucleotide G at positions 176, 875, 2811, 3719 of SEQ ID No 3; and a nucleotide T at positions 2282, and 2549 of SEQ ID No 3. Additional preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1619, 1850, and 2126; a nucleotide C at positions 2062, 3505, and 3823; a nucleotide G at positions 709, 1971, 2059, 2060, 2061, 2107, 2140, and 3824; and a nucleotide T at positions 2533, and

2809 of SEQ ID No 3. Other preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 3: 1355-1357, 1892-1894, 2090-2094, 2530-2532, 2619-2622, 3149-3152, 3183-3187, 3500-3501, 3508-3514, 3527-3532, 3533-3538, 3552-3557, 3746-3749, 3789-3792, 3797-3799, 3809-3815, 3912-3914 and 4056-4058.

An additional object of the invention is a purified, isolated, or recombinant polynucleotide comprising the nucleotide sequence of SEQ ID No 4, complementary sequences thereto, as well as allelic variants, and fragments thereof. Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 4. Additional preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527 and 2460 of SEQ ID No 4; a nucleotide C at position 1013 of SEQ ID No 4; and a nucleotide G at positions 176 and 749 of SEQ ID No 4. Additionally preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708 and 807 and a nucleotide G at position 709 of SEQ ID No 4. Other preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises the pairs of nucleotide positions 1136-1137 of SEQ ID No 4.

One more object of the invention is a purified, isolated, or recombinant polynucleotide comprising the nucleotide sequence of SEQ ID No 8, complementary sequences thereto, as well as allelic variants, and fragments thereof. Particularly preferred nucleic acids of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at

least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 8 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 8: 1-500, 501-1000, 1001-1500, and 1501-1738.

Thus, the invention also relates to nucleic acid probes characterized in that they hybridize specifically, under the stringent hybridization conditions defined above, with a nucleic acid selected from the group consisting of the nucleotide sequences:

a) 1-70715, 70795-82207, 82297-83612, 83824-85297, 85418-86388, 86446-87495, 87523-88294, 88384-89483, 89650-92748, 97156-98309, 98476-99329, 99491-100026, 100212-100281, 100396-100538, 100682-100833, 100995-101920, 102087-102970, 103264-103724, and 103753-106746 of SEQ ID No 1 or a variant thereof or a sequence complementary thereto;

b) 1-162 of SEQ ID No 2 or a variant thereof or a sequence complementary thereto;

c) 1-162 and 747-872 of SEQ ID No 3 or a variant thereof or a sequence complementary thereto;

d) 1-162 of SEQ ID No 4 or a variant thereof or a sequence complementary thereto; and

e) SEQ ID No 8 or a variant thereof or a sequence complementary thereto.

In a preferred embodiment, the oligonucleotides of the invention can hybridize with at least a portion of an intron or of the regulatory sequences of the *PCTA-1* gene. Particularly preferred oligonucleotides of the invention hybridize with a sequence comprised in an intron or in the regulatory sequences of the *PCTA-1* gene. In an other preferred embodiment, the oligonucleotides of the invention can hybridize with at least a portion of an exon selected from the group of exons 0, 1, 6bis, 9, and 9ter.

The present invention also concerns oligonucleotides and groups of oligonucleotides for the detection of alleles of biallelic markers of the *PCTA-1* gene, preferably those associated with cancer, preferably with prostate cancer, with an early onset of prostate cancer, with a susceptibility to prostate cancer, with the level of aggressiveness of prostate cancer tumors, with a modified or forthcoming expression of the *PCTA-1* gene, with a modified or forthcoming production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein. These oligonucleotides are characterized in that they can hybridize with a *PCTA-1* gene, preferably with a polymorphic *PCTA-1* gene and more preferably with a region of a *PCTA-1* gene comprising a polymorphic site containing a specific allele associated with prostate cancer, with the level of aggressiveness of prostate cancer tumors or with modifications in the regulation of expression of the *PCTA-1* gene. These oligonucleotides are useful either as primers for use in

various processes such as DNA amplification and microsequencing or as probes for DNA recognition in hybridization analyses.

Therefore, another preferred embodiment of a probe according to the invention consists of a nucleic acid comprising a biallelic marker selected from the group consisting of A1 to A125 or the complements thereof, for which the respective locations in the sequence listing are provided in Table 2. In some embodiments, the oligonucleotides comprise the polymorphic base of a sequence selected from P1 to P125, and the complementary sequences thereto. In other embodiments, the oligonucleotides have a 3' terminus immediately adjacent to a polymorphic base in the *PCTA-1* gene, such as a polymorphic base comprised in one of the sequences P1 to P125, and the complementary sequence thereto. In other embodiments, the oligonucleotide is capable of discriminating between different alleles of a biallelic marker in the *PCTA-1* gene, including the biallelic markers A1 to A125 and the complements thereof.

In one embodiment the invention encompasses isolated, purified, and recombinant polynucleotides consisting of, or consisting essentially of a contiguous span of 8 to 50 nucleotides of any one of SEQ ID Nos 1, 2, 3, 4 and the complement thereof, wherein said span includes a *PCTA-1*-related biallelic marker in said sequence; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said contiguous span is 18 to 47 nucleotides in length and said biallelic marker is within 4 nucleotides of the center of said polynucleotide; optionally, wherein said polynucleotide consists of said contiguous span and said contiguous span is 25 nucleotides in length and said biallelic marker is at the center of said polynucleotide; optionally, wherein said polynucleotide consists of said contiguous span and said contiguous span is 47 nucleotides in length and said biallelic marker is at the center of said polynucleotide; optionally, wherein the 3' end of said contiguous span is present at the 3' end of said polynucleotide; and optionally, wherein the 3' end of said

contiguous span is located at the 3' end of said polynucleotide and said biallelic marker is present at the 3' end of said polynucleotide. In a preferred embodiment, said probes comprises, consists of, or consists essentially of a sequence selected from the following sequences: P1 to P125 and the complementary sequences thereto.

In another embodiment the invention encompasses isolated, purified and recombinant polynucleotides comprising, consisting of, or consisting essentially of a contiguous span of 8 to 50 nucleotides of SEQ ID Nos 1, 2, 3, 4, or the complements thereof, wherein the 3' end of said contiguous span is located at the 3' end of said polynucleotide, and wherein the 3' end of said polynucleotide is located within 20 nucleotides upstream of a *PCTA-1*-related biallelic marker in said sequence; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein the 3' end of said polynucleotide is located 1 nucleotide upstream of said *PCTA-1*-related biallelic marker in said sequence; and optionally, wherein said polynucleotide consists essentially of a sequence selected from the following sequences: D1 to D125 and E1 to E125.

In a further embodiment, the invention encompasses isolated, purified, or recombinant polynucleotides comprising, consisting of, or consisting essentially of a sequence selected from the following sequences: B1 to B47 and C1 to C47.

In an additional embodiment, the invention encompasses polynucleotides for use in hybridization assays, sequencing assays, and enzyme-based mismatch detection assays for determining the identity of the nucleotide at a *PCTA-1*-related biallelic marker in SEQ ID Nos 1, 2, 3, 4, or the complements thereof, as well as polynucleotides for use in amplifying segments of nucleotides comprising a *PCTA-1*-related biallelic marker in SEQ ID Nos 1, 2, 3, 4, or the complements thereof; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic

markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith.

The formation of stable hybrids depends on the melting temperature (Tm) of the DNA. The Tm depends on the length of the primer or probe, the ionic strength of the solution and the G+C content. The higher the G+C content of the primer or probe, the higher is the melting temperature because G:C pairs are held by three H bonds whereas A:T pairs have only two. The GC content in the probes of the invention usually ranges between 10 and 75 %, preferably between 35 and 60 %, and more preferably between 40 and 55 %.

A probe or a primer according to the invention has between 8 and 1000 nucleotides in length, or is specified to be at least 12, 15, 18, 20, 25, 35, 40, 50, 60, 70, 80, 100, 250, 500 or 1000 nucleotides in length. More particularly, the length of these probes and primers can range from 8, 10, 15, 20, or 30 to 100 nucleotides, preferably from 10 to 50, more preferably from 15 to 30 nucleotides. Shorter probes and primers tend to lack specificity for a target nucleic acid sequence and generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. Longer probes and primers are expensive to produce and can sometimes self-hybridize to form hairpin structures. The appropriate length for primers and probes under a particular set of assay conditions may be empirically determined by one of skill in the art. A preferred probe or primer consists of a nucleic acid comprising a polynucleotide selected from the group of the nucleotide sequences of P1 to P125 and the complementary sequence thereto, B1 to B47, C1 to C47, D1 to D125, E1 to E125, for which the respective locations in the sequence listing are provided in Tables 1, 2, 3 and 4.

The primers and probes can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphodiester method of Narang et al.(1979), the phosphodiester method of Brown et al.(1979), the diethylphosphoramidite method of Beaucage et al.(1981) and the

solid support method described in EP 0 707 592, the disclosure of which is incorporated herein by reference in its entirety.

Detection probes are generally nucleic acid sequences or uncharged nucleic acid analogs such as, for example peptide nucleic acids which are disclosed in International Patent Application WO 92/20702, morpholino analogs which are described in U.S. Patents Numbered 5,185,444; 5,034,506 and 5,142,047, the disclosures of which are incorporated herein by reference in their entireties. The probe may have to be rendered "non-extendable" in that additional dNTPs cannot be added to the probe. In and of themselves analogs usually are non-extendable and nucleic acid probes can be rendered non-extendable by modifying the 3' end of the probe such that the hydroxyl group is no longer capable of participating in elongation. For example, the 3' end of the probe can be functionalized with the capture or detection label to thereby consume or otherwise block the hydroxyl group. Alternatively, the 3' hydroxyl group simply can be cleaved, replaced or modified, U.S. Patent Application Serial No. 07/049,061 filed April 19, 1993 describes modifications, which can be used to render a probe non-extendable.

Any of the polynucleotides of the present invention can be labeled, if desired, by incorporating any label known in the art to be detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive substances (including, ^{32}P, ^{35}S, ^{3}H, ^{125}I), fluorescent dyes (including, 5-bromodesoxyuridin, fluorescein, acetylaminofluorene, digoxigenin) or biotin. Preferably, polynucleotides are labeled at their 3' and 5' ends. Examples of non-radioactive labeling of nucleic acid fragments are described in the French patent No. FR-7810975 or by Urdea et al (1988) or Sanchez-Pescador et al (1988), the disclosures of which are incorporated herein by reference in their entireties. In addition, the probes according to the present invention may have structural characteristics such that they allow the signal amplification, such structural characteristics being, for example, branched DNA probes as those described by Urdea et al. in 1991 or in the European patent No. EP 0 225 807 (Chiron), the disclosures of which are incorporated herein by reference in their entireties.

A label can also be used to capture the primer, so as to facilitate the immobilization of either the primer or a primer extension product, such as amplified DNA, on a solid support. A capture label is attached to the primers or probes and can be a specific binding member which forms a binding pair with the solid's phase reagent's specific binding member (e.g. biotin and streptavidin). Therefore depending upon the type of label carried by a polynucleotide or a probe, it may be employed to capture or to detect the target DNA. Further, it will be understood that the polynucleotides, primers or probes provided herein, may, themselves, serve as the

capture label. For example, in the case where a solid phase reagent's binding member is a nucleic acid sequence, it may be selected such that it binds a complementary portion of a primer or probe to thereby immobilize the primer or probe to the solid phase. In cases where a polynucleotide probe itself serves as the binding member, those skilled in the art will recognize that the probe will contain a sequence or "tail" that is not complementary to the target. In the case where a polynucleotide primer itself serves as the capture label, at least a portion of the primer will be free to hybridize with a nucleic acid on a solid phase. DNA Labeling techniques are well known to the skilled technician.

The probes of the present invention are useful for a number of purposes. They can be notably used in Southern hybridization to genomic DNA. The probes can also be used to detect PCR amplification products. They may also be used to detect mismatches in the *PCTA-1* gene or mRNA using other techniques.

Any of the polynucleotides, primers and probes of the present invention can be conveniently immobilized on a solid support. Solid supports are known to those skilled in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, sheep (or other animal) red blood cells, duracytes and others. The solid support is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads, membranes, plastic tubes, walls of microtiter wells, glass or silicon chips, sheep (or other suitable animal's) red blood cells and duracytes are all suitable examples. Suitable methods for immobilizing nucleic acids on solid phases include ionic, hydrophobic, covalent interactions and the like. A solid support, as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid support can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid support and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid support material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, sheep (or other suitable animal's) red blood cells, duracytes® and other configurations known to those of ordinary skill in the art.

The polynucleotides of the invention can be attached to or immobilized on a solid support individually or in groups of at least 2, 5, 8, 10, 12, 15, 20, or 25 distinct polynucleotides of the invention to a single solid support. In addition, polynucleotides other than those of the invention may be attached to the same solid support as one or more polynucleotides of the invention.

Consequently, the invention also deals with a method for detecting the presence of a nucleic acid comprising a nucleotide sequence selected from a group consisting of SEQ ID Nos 1, 2, 3, 4, 8, a fragment or a variant thereof and a complementary sequence thereto in a sample, said method comprising the following steps of:

a) bringing into contact a nucleic acid probe or a plurality of nucleic acid probes which can hybridize with a nucleotide sequence included in a nucleic acid selected form the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8, a fragment or a variant thereof and a complementary sequence thereto and the sample to be assayed; and

b) detecting the hybrid complex formed between the probe and a nucleic acid in the sample.

The invention further concerns a kit for detecting the presence of a nucleic acid comprising a nucleotide sequence selected from a group consisting of SEQ ID Nos 1, 2, 3, 4, 8, a fragment or a variant thereof and a complementary sequence thereto in a sample, said kit comprising:

a) a nucleic acid probe or a plurality of nucleic acid probes which can hybridize with a nucleotide sequence included in a nucleic acid selected form the group consisting of the nucleotide sequences of SEQ ID Nos 1, 2, 3, 4, 8, a fragment or a variant thereof and a complementary sequence thereto; and

b) optionally, the reagents necessary for performing the hybridization reaction.

In a first preferred embodiment of this detection method and kit, said nucleic acid probe or the plurality of nucleic acid probes are labeled with a detectable molecule. In a second preferred embodiment of said method and kit, said nucleic acid probe or the plurality of nucleic acid probes has been immobilized on a substrate. In a third preferred embodiment, the nucleic acid probe or the plurality of nucleic acid probes comprise either a sequence which is selected from the group consisting of the nucleotide sequences of P1 to P125 and the complementary sequence thereto, B1 to B47, C1 to C47, D1 to D125, E1 to E125 or a biallelic marker selected from the group consisting of A1 to A125 and the complements thereto.

Oligonucleotide Arrays

A substrate comprising a plurality of oligonucleotide primers or probes of the invention may be used either for detecting or amplifying targeted sequences in the *PCTA-1* gene and may also be used for detecting mutations in the coding or in the non-coding sequences of the *PCTA-1* gene.

Any polynucleotide provided herein may be attached in overlapping areas or at random locations on the solid support. Alternatively the polynucleotides of the invention may be attached in an ordered array wherein each polynucleotide is attached to a distinct region of the solid support which does not overlap with the attachment site of any other polynucleotide. Preferably, such an ordered array of polynucleotides is designed to be "addressable" where the distinct locations are recorded and can be accessed as part of an assay procedure. Addressable polynucleotide arrays typically comprise a plurality of different oligonucleotide probes that are coupled to a surface of a substrate in different known locations. The knowledge of the precise location of each polynucleotides location makes these "addressable" arrays particularly useful in hybridization assays. Any addressable array technology known in the art can be employed with the polynucleotides of the invention. One particular embodiment of these polynucleotide arrays is known as the Genechips™, and has been generally described in US Patent 5,143,854; PCT publications WO 90/15070 and 92/10092, the disclosures of which are incorporated herein by reference in their entireties. These arrays may generally be produced using mechanical synthesis methods or light directed synthesis methods which incorporate a combination of photolithographic methods and solid phase oligonucleotide synthesis (Fodor et al., 1991). The immobilization of arrays of oligonucleotides on solid supports has been rendered possible by the development of a technology generally identified as "Very Large Scale Immobilized Polymer Synthesis" (VLSIPS™) in which, typically, probes are immobilized in a high density array on a solid surface of a chip. Examples of VLSIPS™ technologies are provided in US Patents 5,143,854; and 5,412,087 and in PCT Publications WO 90/15070, WO 92/10092 and WO 95/11995, the disclosures of which are incorporated herein by reference in their entireties, which describe methods for forming oligonucleotide arrays through techniques such as light-directed synthesis techniques. In designing strategies aimed at providing arrays of nucleotides immobilized on solid supports, further presentation strategies were developed to order and display the oligonucleotide arrays on the chips in an attempt to maximize hybridization patterns and sequence information. Examples of such presentation strategies are disclosed in PCT Publications WO 94/12305, WO 94/11530, WO 97/29212 and WO 97/31256.

In another embodiment of the oligonucleotide arrays of the invention, an oligonucleotide probe matrix may advantageously be used to detect mutations occurring in the

PCTA-1 gene and preferably in its regulatory region. For this particular purpose, probes are specifically designed to have a nucleotide sequence allowing their hybridization to the genes that carry known mutations (either by deletion, insertion or substitution of one or several nucleotides). By known mutations, it is meant, mutations on the *PCTA-1* gene that have been identified according, for example to the technique used by Huang et al.(1996) or Samson et al.(1996).

Another technique that is used to detect mutations in the *PCTA-1* gene is the use of a high-density DNA array. Each oligonucleotide probe constituting a unit element of the high density DNA array is designed to match a specific subsequence of the *PCTA-1* genomic DNA or cDNA. Thus, an array consisting of oligonucleotides complementary to subsequences of the target gene sequence is used to determine the identity of the target sequence with the wild gene sequence, measure its amount, and detect differences between the target sequence and the reference wild gene sequence of the *PCTA-1* gene. In one such design, termed 4L tiled array, is implemented a set of four probes (A, C, G, T), preferably 15-nucleotide oligomers. In each set of four probes, the perfect complement will hybridize more strongly than mismatched probes. Consequently, a nucleic acid target of length L is scanned for mutations with a tiled array containing 4L probes, the whole probe set containing all the possible mutations in the known wild reference sequence. The hybridization signals of the 15-mer probe set tiled array are perturbed by a single base change in the target sequence. As a consequence, there is a characteristic loss of signal or a "footprint" for the probes flanking a mutation position. This technique was described by Chee et al. in 1996.

Consequently, the invention concerns an array of nucleic acid molecules comprising at least one polynucleotide described above as probes and primers. Preferably, the invention concerns an array of nucleic acid comprising at least two polynucleotides described above as probes and primers.

A further object of the invention consists of an array of nucleic acid sequences comprising either at least one of the sequences selected from the group consisting of P1 to P125, B1 to B47, C1 to C47, D1 to D125, E1 to E125, the sequences complementary thereto, a fragment thereof of at least 8, 10, 12, 15, 18, 20, 25, 30, or 40 consecutive nucleotides thereof, and at least one sequence comprising a biallelic marker selected from the group consisting of A1 to A125 and the complements thereto.

The invention also pertains to an array of nucleic acid sequences comprising either at least two of the sequences selected from the group consisting of P1 to P125, B1 to B47, C1 to C47, D1 to D125, E1 to E125, the sequences complementary thereto, a fragment thereof of at

least 8 consecutive nucleotides thereof, and at least two sequences comprising a biallelic marker selected from the group consisting of A1 to A125 and the complements thereof.

PCTA-1 Proteins And Polypeptide Fragments Thereof

The term "PCTA-1 polypeptides" is used herein to embrace all of the proteins and polypeptides of the present invention. Also forming part of the invention are polypeptides encoded by the polynucleotides of the invention, as well as fusion polypeptides comprising such polypeptides.

The invention embodies PCTA-1 proteins from humans, including isolated or purified PCTA-1 proteins consisting, consisting essentially, or comprising the sequence of SEQ ID No 5. It should be noted the PCTA-1 proteins of the invention are based on the naturally-occurring variant of the amino acid sequence of human PCTA-1, wherein the valine residue of amino acid position 170 has been replaced with a serine residue and the glutamine residue of amino acid position 203 has been replaced with a lysine residue. This variant protein and the fragments thereof which contain a serine at the amino acid position 170 and a lysine at the amino acid position 203 of SEQ ID No 5 are collectively referred to herein as "170-Ser 203-Lys variants." In another embodiment, the present invention concerns a purified and/or isolated nucleic acid encoding the PCTA-1 protein of SEQ ID No 5 or variant or fragment thereof.

The invention also concerns a purified and/or isolated PCTA-1 protein comprising a sequence selected from the group consisting of SEQ ID Nos 6, 7 and variants and functional fragments thereof. In another embodiment, the present invention concerns a purified and/or isolated nucleic acid encoding the PCTA-1 protein comprising a sequence selected from the group consisting of SEQ ID Nos 6, 7 or a variant or a fragment thereof.

The invention also encompasses the amino acid sequence of a murine PCTA-1 protein, such as that of SEQ ID No 9, fragments and variants thereof. The invention also concerns a nucleotide sequence encoding the murine PCTA-1 protein of SEQ ID No 9, sequences complementary thereto and fragments and variants thereof.

The invention also relates to modified human and mouse PCTA-1 proteins and to fragments and variants thereof. The term "modified PCTA-1 protein" is intended to designate a PCTA-1 protein which, when compared to a native PCTA-1 protein of SEQ ID No 5, 6, or 7, bears at least one amino acid substitution, deletion or addition. More particularly, preferred modified PCTA-1 proteins include the proteins bearing at least one of the following amino acid substitutions:

- a substitution from F to Y at position 18, a substitution from R to C at position 35, a substitution from V to M at position 55 and a substitution from S to R at position 183 in SEQ ID No 5;

- a substitution from F to Y at position 18, a substitution from R to C at position 35, a substitution from V to M at position 55, a substitution from D to Y at position 204 and a substitution from S to R at position 225 in SEQ ID No 6; and

- a substitution from F to Y at position 18, a substitution from R to C at position 35, a substitution from V to M at position 55 and a substitution from S to R at position 183 in SEQ ID No 7.

Modified proteins bearing two or more of such substitutions also fall within the scope of the present invention. Other preferred embodiments include regions of the modified PCTA-1 proteins of the invention, and particularly those regions bearing at least one of the substitutions described above. Particularly preferred regions are those possessing antigenic properties and which can be used in vaccine agents or to raise antibodies against the PCTA-1 protein, and which most preferably comprise at least one of the particular substitutions referred to above.

The term "modified PCTA-1 protein" also designates a truncated PCTA-1 protein consisting of the amino acid sequence 1-211 of SEQ ID No 7.

The present invention embodies isolated, purified, and recombinant polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5.

The present invention embodies isolated, purified, and recombinant polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

- at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6.

The present invention embodies isolated, purified, and recombinant polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7, wherein said contiguous span includes:

- a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

- at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

- at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7.

The invention also concerns the truncated PCTA-1 protein consisting essentially of or consisting of the amino acid positions 1-211 of SEQ ID No 7.

In other preferred embodiments the contiguous stretch of amino acids from SEQ ID Nos 5, 6, 7 comprises the site of a mutation or functional mutation, including a deletion, addition, swap or truncation of the amino acids in the *PCTA-1* protein sequence.

The present invention embodies isolated, purified, and recombinant polypeptides comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9.

PCTA-1 proteins are preferably isolated from human or mammalian tissue samples or expressed from human or mammalian genes. The PCTA-1 polypeptides of the invention can be made using routine expression methods known in the art. The polynucleotide encoding the desired polypeptide, is ligated into an expression vector suitable for any convenient host. Both eukaryotic and prokaryotic host systems is used in forming recombinant polypeptides, and a summary of some of the more common systems. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use. Purification is by any technique known in the art, for example, differential extraction, salt fractionation, chromatography, centrifugation, and the like. See, for example, Methods in Enzymology for a variety of methods for purifying proteins.

In addition, shorter protein fragments is produced by chemical synthesis. Alternatively the proteins of the invention is extracted from cells or tissues of humans or non-human animals. Methods for purifying proteins are known in the art, and include the use of detergents or chaotropic agents to disrupt particles followed by differential extraction and separation of the

polypeptides by ion exchange chromatography, affinity chromatography, sedimentation according to density, and gel electrophoresis.

Any *PCTA-1* cDNA, including SEQ ID Nos 2, 3, 4, 8, is used to express PCTA-1 proteins and polypeptides. The nucleic acid encoding a PCTA-1 protein or polypeptide to be expressed is operably linked to a promoter in an expression vector using conventional cloning technology. The *PCTA-1* insert in the expression vector may comprise the full coding sequence for a PCTA-1 protein or a fragment thereof. For example, the *PCTA-1* derived insert may encode a polypeptide as described above.

The expression vector is any of the mammalian, yeast, insect or bacterial expression systems known in the art. Commercially available vectors and expression systems are available from a variety of suppliers including Genetics Institute (Cambridge, MA), Stratagene (La Jolla, California), Promega (Madison, Wisconsin), and Invitrogen (San Diego, California). If desired, to enhance expression and facilitate proper protein folding, the codon context and codon pairing of the sequence is optimized for the particular expression organism in which the expression vector is introduced, as explained by Hatfield, et al., U.S. Patent No. 5,082,767, the disclosure of which is incorporated herein by reference in its entirety.

In one embodiment, the entire coding sequence of a *PCTA-1* cDNA through the poly A signal of the cDNA are operably linked to a promoter in the expression vector. Alternatively, if the nucleic acid encoding a fragment of the PCTA-1 protein lacks a methionine to serve as the initiation site, an initiating methionine can be introduced next to the first codon of the nucleic acid using conventional techniques. Similarly, if the insert from a *PCTA-1* cDNA lacks a poly A signal, this sequence can be added to the construct by, for example, splicing out the Poly A signal from pSG5 (Stratagene) using BglI and SalI restriction endonuclease enzymes and incorporating it into the mammalian expression vector pXT1 (Stratagene). pXT1 contains the LTRs and a portion of the gag gene from Moloney Murine Leukemia Virus. The position of the LTRs in the construct allow efficient stable transfection. The vector includes the Herpes Simplex Thymidine Kinase promoter and the selectable neomycin gene. The nucleic acid encoding a PCTA-1 protein or a fragment thereof is obtained by PCR from a bacterial vector containing a *PCTA-1* cDNA selected from the group consisting of SEQ ID Nos 2, 3,4, and 8 using oligonucleotide primers complementary to the *PCTA-1* cDNA or fragment thereof and containing restriction endonuclease sequences for Pst I incorporated into the 5′primer and BglII at the 5′ end of the corresponding cDNA 3′ primer, taking care to ensure that the sequence encoding the PCTA-1 protein or a fragment thereof is positioned properly with respect to the poly A signal. The purified fragment obtained from the resulting PCR reaction is digested with PstI, blunt ended with an exonuclease,

digested with Bgl II, purified and ligated to pXT1, now containing a poly A signal and digested with BglII.

The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc., Grand Island, New York) under conditions outlined in the product specification. Positive transfectants are selected after growing the transfected cells in 600ug/ml G418 (Sigma, St. Louis, Missouri).

Alternatively, the nucleic acids encoding the *PCTA-1* protein or a fragment thereof is cloned into pED6dpc2 (Genetics Institute, Cambridge, MA). The resulting pED6dpc2 constructs is transfected into a suitable host cell, such as COS 1 cells. Methotrexate resistant cells are selected and expanded.

The above procedures may also be used to express a mutant PCTA-1 protein responsible for a detectable phenotype or a fragment thereof.

The expressed proteins is purified using conventional purification techniques such as ammonium sulfate precipitation or chromatographic separation based on size or charge. The protein encoded by the nucleic acid insert may also be purified using standard immunochromatography techniques. In such procedures, a solution containing the expressed PCTA-1 protein or fragment thereof, such as a cell extract, is applied to a column having antibodies against the PCTA-1 protein or fragment thereof is attached to the chromatography matrix. The expressed protein is allowed to bind the immunochromatography column. Thereafter, the column is washed to remove non-specifically bound proteins. The specifically bound expressed protein is then released from the column and recovered using standard techniques.

To confirm expression of a PCTA-1 protein or a fragment thereof, the proteins expressed from host cells containing an expression vector containing an insert encoding a PCTA-1 protein or a fragment thereof can be compared to the proteins expressed in host cells containing the expression vector without an insert. The presence of a band in samples from cells containing the expression vector with an insert which is absent in samples from cells containing the expression vector without an insert indicates that the PCTA-1 protein or a fragment thereof is being expressed. Generally, the band will have the mobility expected for the PCTA-1 protein or fragment thereof. However, the band may have a mobility different than that expected as a result of modifications such as glycosylation, ubiquitination, or enzymatic cleavage.

Antibodies capable of specifically recognizing the expressed PCTA-1 protein or a fragment thereof are described below.

If antibody production is not possible, the nucleic acids encoding the PCTA-1 protein or a fragment thereof is incorporated into expression vectors designed for use in purification schemes employing chimeric polypeptides. In such strategies the nucleic acid encoding the PCTA-1 protein

or a fragment thereof is inserted in frame with the gene encoding the other half of the chimera. The other half of the chimera is β-globin or a nickel binding polypeptide encoding sequence. A chromatography matrix having antibody to β-globin or nickel attached thereto is then used to purify the chimeric protein. Protease cleavage sites is engineered between the β-globin gene or the nickel binding polypeptide and the PCTA-1 protein or fragment thereof. Thus, the two polypeptides of the chimera is separated from one another by protease digestion.

One useful expression vector for generating β-globin chimeric proteins is pSG5 (Stratagene), which encodes rabbit β-globin. Intron II of the rabbit β-globin gene facilitates splicing of the expressed transcript, and the polyadenylation signal incorporated into the construct increases the level of expression. These techniques are well known to those skilled in the art of molecular biology. Standard methods are published in methods texts such as Davis et al., (1986) and many of the methods are available from Stratagene, Life Technologies, Inc., or Promega. Polypeptide may additionally be produced from the construct using in vitro translation systems such as the In vitro Express™ Translation Kit (Stratagene).

Antibodies That Bind PCTA-1 Polypeptides of the Invention

Any PCTA-1 polypeptide or whole protein may be used to generate antibodies capable of specifically binding to an expressed PCTA-1 protein or fragments thereof as described.

One antibody composition of the invention is capable of specifically binding or specifically bind to the 170-Ser 203-Lys variant of the PCTA-1 protein of SEQ ID No 5. An other antibody composition of the invention is capable of specifically binding or specifically bind to the PCTA-1 protein selected from the group consisting of amino acid sequences of SEQ ID Nos 6, 7, 9. For an antibody composition to specifically bind to a first variant of PCTA-1, it must demonstrate at least a 5%, 10%, 15%, 20%, 25%, 50%, or 100% greater binding affinity for a full length first variant of the PCTA-1 protein than for a full length second variant of the PCTA-1 protein in an ELISA, RIA, or other antibody-based binding assay.

In a preferred embodiment, the invention concerns antibody compositions, either polyclonal or monoclonal, capable of selectively binding, or selectively bind to an epitope-containing any one of the following polypeptides:

a) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5 , wherein said epitope comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5;

b) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6 , wherein said epitope comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6;

c) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7 , wherein said epitope comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7; and

d) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9.

The invention also concerns a purified or isolated antibody capable of specifically binding to a mutated PCTA-1 protein or to a fragment or variant thereof comprising an epitope of the mutated PCTA-1 protein. In another preferred embodiment, the present invention concerns an antibody capable of binding to a polypeptide comprising at least 10 consecutive

amino acids of a PCTA-1 protein and including at least one of the amino acids which can be encoded by the trait causing mutations.

In a preferred embodiment, the invention concerns the use of any one of the following polypeptides in the manufacture of antibodies:

a) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5 , wherein said contigous span comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5;

b) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6 , wherein said contigous span comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

i) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

ii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6;

c) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7 , wherein said contigous span comprises:

) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

i) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

ii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7; and

d) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9.

Non-human animals or mammals, whether wild-type or transgenic, which express a different species of PCTA-1 than the one to which antibody binding is desired, and animals which do not express *PCTA-1* (i.e. a *PCTA-1* knock out animal as described in herein) are particularly useful for preparing antibodies. *PCTA-1* knock out animals will recognize all or most of the exposed regions of a PCTA-1 protein as foreign antigens, and therefore produce antibodies with a wider array of PCTA-1 epitopes. Moreover, smaller polypeptides with only 10 to 30 amino acids may be useful in obtaining specific binding to any one of the PCTA-1 proteins. In addition, the humoral immune system of animals which produce a species of PCTA-1 that resembles the antigenic sequence will preferentially recognize the differences between the animal's native PCTA-1 species and the antigen sequence, and produce antibodies to these unique sites in the antigen sequence. Such a technique will be particularly useful in obtaining antibodies that specifically bind to any one of the PCTA-1 proteins.

Antibody preparations prepared according to either protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample. The antibodies may also be used in therapeutic compositions for killing cells expressing the protein or reducing the levels of the protein in the body.

The antibodies of the invention may be labeled by any one of the radioactive, fluorescent or enzymatic labels known in the art.

Consequently, the invention is also directed to a method for detecting specifically the presence of a PCTA-1 polypeptide according to the invention in a biological sample, said method comprising the following steps :

a) bringing into contact the biological sample with a polyclonal or monoclonal antibody that specifically binds a PCTA-1 polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID Nos 5, 6, 7, 9, or to a peptide fragment or variant thereof; and

b) detecting the antigen-antibody complex formed.

The invention also concerns a diagnostic kit for detecting *in vitro* the presence of a PCTA-1 polypeptide according to the present invention in a biological sample, wherein said kit comprises:

a) a polyclonal or monoclonal antibody that specifically binds a PCTA-1 polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID Nos 5, 6, 7, 9, or to a peptide fragment or variant thereof, optionally labeled;

b) a reagent allowing the detection of the antigen-antibody complexes formed, said reagent carrying optionally a label, or being able to be recognized itself by a labeled reagent, more particularly in the case when the above-mentioned monoclonal or polyclonal antibody is not labeled by itself.

PCTA-1-Related Biallelic markers

Advantages Of The Biallelic Markers Of The Present Invention

The *PCTA-1*-related biallelic markers of the present invention offer a number of important advantages over other genetic markers such as RFLP (Restriction fragment length polymorphism) and VNTR (Variable Number of Tandem Repeats) markers.

The first generation of markers, were RFLPs, which are variations that modify the length of a restriction fragment. But methods used to identify and to type RFLPs are relatively wasteful of materials, effort, and time. The second generation of genetic markers were VNTRs, which can be categorized as either minisatellites or microsatellites. Minisatellites are tandemly repeated DNA sequences present in units of 5-50 repeats which are distributed along regions of the human chromosomes ranging from 0.1 to 20 kilobases in length. Since they present many possible alleles, their informative content is very high. Minisatellites are scored by performing Southern blots to identify the number of tandem repeats present in a nucleic acid sample from the individual being tested. However, there are only 10^4 potential VNTRs that can be typed by Southern blotting. Moreover, both RFLP and VNTR markers are costly and time-consuming to develop and assay in large numbers.

Single nucleotide polymorphism or biallelic markers can be used in the same manner as RFLPs and VNTRs but offer several advantages. SNP are densely spaced in the human genome and represent the most frequent type of variation. An estimated number of more than 10^7 sites are scattered along the $3x10^9$ base pairs of the human genome. Therefore, SNP occur at a greater frequency and with greater uniformity than RFLP or VNTR markers which means that there is a greater probability that such a marker will be found in close proximity to a genetic locus of interest. SNP are less variable than VNTR markers but are mutationally more stable.

Also, the different forms of a characterized single nucleotide polymorphism, such as the biallelic markers of the present invention, are often easier to distinguish and can therefore be typed easily on a routine basis. Biallelic markers have single nucleotide based alleles and they

have only two common alleles, which allows highly parallel detection and automated scoring. The biallelic markers of the present invention offer the possibility of rapid, high throughput genotyping of a large number of individuals.

Biallelic markers are densely spaced in the genome, sufficiently informative and can be assayed in large numbers. The combined effects of these advantages make biallelic markers extremely valuable in genetic studies. Biallelic markers can be used in linkage studies in families, in allele sharing methods, in linkage disequilibrium studies in populations, in association studies of case-control populations or of trait positive and trait negative populations. An important aspect of the present invention is that biallelic markers allow association studies to be performed to identify genes involved in complex traits. Association studies examine the frequency of marker alleles in unrelated case- and control-populations and are generally employed in the detection of polygenic or sporadic traits. Association studies may be conducted within the general population and are not limited to studies performed on related individuals in affected families (linkage studies). Biallelic markers in different genes can be screened in parallel for direct association with disease or response to a treatment. This multiple gene approach is a powerful tool for a variety of human genetic studies as it provides the necessary statistical power to examine the synergistic effect of multiple genetic factors on a particular phenotype, drug response, sporadic trait, or disease state with a complex genetic etiology.

PCTA-1-Related Biallelic Markers And Polynucleotides Related Thereto

The invention also concerns a purified and/or isolated *PCTA-1*-related biallelic marker located in the sequence of the *PCTA-1* gene, preferably a biallelic marker comprising an allele associated with prostate cancer, with an early onset of prostate cancer, with a response to a prophylactic or therapeutic agent administered for cancer treatment, particularly prostate cancer, with the level of aggressiveness of prostate cancer tumors, with a modified or forthcoming expression of the *PCTA-1* gene, with a modified or forthcoming production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein. The term *PCTA-1*-related biallelic marker includes the biallelic markers designated A1 to A125. The invention also concerns sets of these biallelic markers.

125 biallelic markers were identified. They include 3 deletions, 6 insertions and 2 variable motifs. 40 biallelic markers, namely A45, A54 to A56, A59 to A61, A75, A76, A85, A93 to A122, were located in exonic region. 39 biallelic markers, namely A44, A46 to A53, A57 to A58, A62 to A74, A77 to A84, A86 to A92, were localized in intronic region of the *PCTA-1* gene. 3 biallelic markers A123, A124 and A125 were in the 3' regulatory region. 43

biallelic markers, namely A1 to A43, were located in the 5' regulatory region. More particularly, 16 of them, namely A28 to A43, were in the promoter of the *PCTA-1* gene.

Among the exonic biallelic markers, 6 of them change the amino acid sequence of a PCTA-1 protein. First, the biallelic marker A54 encodes either a residue tyrosine or phenylalanine. The biallelic marker A56 encodes either a residue cysteine or arginine. The marker A60 encodes either a residue valine or methionine. The marker A75 encodes either a residue aspartic acid or tyrosine. The marker A76 encodes either a leucine residue or a STOP. Finally, the biallelic marker A85 encodes either a residue serine or arginine.

The invention also relates to a purified and/or isolated nucleotide sequence comprising a polymorphic base of a *PCTA-1*-related biallelic marker, preferably of a biallelic marker selected from the group consisting of A1 to A125, and the complements thereof. The sequence has between 8 and 1000 nucleotides in length, and preferably comprises at least 8, 10, 12, 15, 18, 20, 25, 35, 40, 50, 60, 70, 80, 100, 250, 500 or 1000 contiguous nucleotides, to the extent that such lengths are consistent with the specific sequence, of a nucleotide sequence selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, or a variant thereof or a complementary sequence thereto. These nucleotide sequences comprise the polymorphic base of either allele 1 or allele 2 of the considered biallelic marker. Optionally, said biallelic marker may be within 6, 5, 4, 3, 2, or 1 nucleotides of the center of said polynucleotide or at the center of said polynucleotide. Optionally, the 3' end of said contiguous span may be present at the 3' end of said polynucleotide. Optionally, biallelic marker may be present at the 3' end of said polynucleotide. Optionally, the 3' end of said polynucleotide may be located within or at least 2, 4, 6, 8, 10, 12, 15, 18, 20, 25, 50, 100, 250, 500, or 1000 nucleotides upstream of a *PCTA-1*-related biallelic marker in said sequence. Optionally, the 3' end of said polynucleotide may be located 1 nucleotide upstream of a *PCTA-1*-related biallelic marker in said sequence. Optionally, said polynucleotide may further comprise a label. Optionally, said polynucleotide can be attached to solid support. In a further embodiment, the polynucleotides defined above can be used alone or in any combination.

In a preferred embodiment, the sequences comprising a polymorphic base of one of the biallelic markers listed in Table 2 are selected from the group consisting of the nucleotide sequences that have a contiguous span of, that consist of, that are comprised in, or that comprises a polynucleotide having one of the sequences set forth as the amplicons listed in Table 1 or a variant thereof or a complementary sequence thereto.

The invention further concerns a nucleic acid encoding a PCTA-1 protein, wherein said nucleic acid comprises a polymorphic base of a biallelic marker selected from the group consisting of A1 to A125 and the complements thereof.

The invention also relates to a purified and/or isolated nucleotide sequence comprising a sequence defining a biallelic marker located in the sequence of the *PCTA-1* gene. Preferably, the sequences defining a biallelic marker include the polymorphic base of one of the sequences P1 to P125 or the complementary sequence thereto. In some embodiments, the sequences defining a biallelic marker comprise one of the sequences selected from the group consisting of P1 to P125, or a fragment or variant thereof or a complementary sequence thereto, said fragment comprising the polymorphic base.

The invention also concerns a set of the purified and/or isolated nucleotide sequences defined above. More particularly, the set of purified and/or isolated nucleotide sequences comprises a group of sequences defining a combination of biallelic markers located in the sequence of the *PCTA-1* gene, preferably wherein alleles of said biallelic markers or the combinations thereof are associated with prostate cancer, with the level of aggressiveness of prostate cancer tumors, or with a level of expression of the *PCTA-1* gene.

In a preferred embodiment, the invention relates to a set of purified and/or isolated nucleotide sequences, each sequence comprising a sequence defining a biallelic marker located in the sequence of the *PCTA-1* gene, wherein the set is characterized in that between about 30 and 100 %, preferably between about 40 and 60 %, more preferably between 50 and 60 %, of the sequences defining a biallelic marker are selected from the group consisting of P1 to P125, or a fragment or variant thereof or a complementary sequence thereto, said fragment comprising the polymorphic base.

More particularly, the invention concerns a set of purified and/or isolated nucleotide sequences, each sequence comprising a sequence defining a different biallelic marker located in the sequence of the *PCTA-1* gene, said biallelic marker being either included in one of the nucleotide sequences of P1 to P125 or a complementary sequence thereto, or a biallelic marker preferably located in the sequence of the *PCTA-1* gene, more preferably biallelic markers A1 to A125 and the complements thereof, and/or in linkage disequilibrium with one of the markers A1 to A125.

The invention also relates to a set of at least two, preferably four, five, six, seven, eight or more nucleotide sequences selected from the group consisting of P1 to P125, or a fragment or variant thereof or a complementary sequence thereto, said fragment comprising the polymorphic base.

The invention further concerns a nucleotide sequence selected from the group consisting of P1 to P125 or a fragment or a variant thereof or a complementary sequence thereto, said fragment comprising the polymorphic base.

The invention also encompasses the use of any polynucleotide for, or any polynucleotide for use in, determining the identity of one or more nucleotides at a *PCTA-1*-related biallelic marker. In addition, the polynucleotides of the invention for use in determining the identity of one or more nucleotides at a *PCTA-1*-related biallelic marker encompass polynucleotides with any further limitation described in this disclosure, or those following, specified alone or in any combination. Optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, said polynucleotide may comprise a sequence disclosed in the present specification; Optionally, said polynucleotide may consist of, or consist essentially of any polynucleotide described in the present specification; Optionally, said determining may be performed in a hybridization assay, a sequencing assay, a microsequencing assay, or an enzyme-based mismatch detection assay; A preferred polynucleotide may be used in a hybridization assay for determining the identity of the nucleotide at a *PCTA-1*-related biallelic marker. Another preferred polynucleotide may be used in a sequencing or microsequencing assay for determining the identity of the nucleotide at a *PCTA-1*-related biallelic marker. A third preferred polynucleotide may be used in an enzyme-based mismatch detection assay for determining the identity of the nucleotide at a *PCTA-1*-related biallelic marker. A fourth preferred polynucleotide may be used in amplifying a segment of polynucleotides comprising a *PCTA-1*-related biallelic marker. Optionally, any of the polynucleotides described above may be attached to a solid support, array, or addressable array; Optionally, said polynucleotide may be labeled.

Additionally, the invention encompasses the use of any polynucleotide for, or any polynucleotide for use in, amplifying a segment of nucleotides comprising a *PCTA-1*-related biallelic marker. In addition, the polynucleotides of the invention for use in amplifying a segment of nucleotides comprising a *PCTA-1*-related biallelic marker encompass

polynucleotides with any further limitation described in this disclosure, or those following, specified alone or in any combination: Optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, said polynucleotide may comprise a sequence disclosed in the present specification; Optionally, said polynucleotide may consist of, or consist essentially of any polynucleotide described in the present specification; Optionally, said amplifying may be performed by a PCR or LCR. Optionally, said polynucleotide may be attached to a solid support, array, or addressable array. Optionally, said polynucleotide may be labeled.

The primers for amplification or sequencing reaction of a polynucleotide comprising a biallelic marker of the invention may be designed from the disclosed sequences for any method known in the art. A preferred set of primers are fashioned such that the 3' end of the contiguous span of identity with a sequence selected from the group consisting of SEQ ID Nos 1, 2, 3, 4 or a sequence complementary thereto or a variant thereof is present at the 3' end of the primer. Such a configuration allows the 3' end of the primer to hybridize to a selected nucleic acid sequence and dramatically increases the efficiency of the primer for amplification or sequencing reactions. Allele specific primers may be designed such that a polymorphic base of a biallelic marker is at the 3' end of the contiguous span and the contiguous span is present at the 3' end of the primer. Such allele specific primers tend to selectively prime an amplification or sequencing reaction so long as they are used with a nucleic acid sample that contains one of the two alleles present at a biallelic marker. The 3' end of the primer of the invention may be located within or at least 2, 4, 6, 8, 10, 12, 15, 18, 20, 25, 50, 100, 250, 500, or 1000 nucleotides upstream of a *PCTA-1*-related biallelic marker in said sequence or at any other location which is appropriate for their intended use in sequencing, amplification or the location of novel sequences or markers. Thus, another set of preferred amplification primers comprise an isolated

polynucleotide consisting essentially of a contiguous span of 8 to 50 nucleotides in a sequence selected from the group consisting of SEQ ID Nos 1, 2, 3, 4 or a sequence complementary thereto or a variant thereof, wherein the 3' end of said contiguous span is located at the 3'end of said polynucleotide, and wherein the 3'end of said polynucleotide is located upstream of a *PCTA-1*-related biallelic marker in said sequence. Preferably, those amplification primers comprise a sequence selected from the group consisting of the sequences B1 to B47 and C1 to C47. Primers with their 3' ends located 1 nucleotide upstream of a biallelic marker of *PCTA-1* have a special utility as microsequencing assays. Preferred microsequencing primers are described in Table 4. Optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, microsequencing primers are selected from the group consisting of the nucleotide sequences D1 to D125 and E1 to E125. More preferred microsequencing primers are selected from the group consisting of the nucleotides sequences D15, D24, D30, D34, D36, D38, D41, D44, D50, D53, D54, D56, D57, D59, D76, D85, D93, D108, D111, D115, D124, E11, E14, E22, E25, E26, E35, E42, E52, E53, E55, E56, E60, E61, E64, E73, E75, E93, E96.

The probes of the present invention may be designed from the disclosed sequences for any method known in the art, particularly methods which allow for testing if a marker disclosed herein is present. A preferred set of probes may be designed for use in the hybridization assays of the invention in any manner known in the art such that they selectively bind to one allele of a biallelic marker, but not the other under any particular set of assay conditions. Preferred hybridization probes comprise the polymorphic base of either allele 1 or allele 2 of the considered biallelic marker. Optionally, said biallelic marker may be within 6, 5, 4, 3, 2, or 1 nucleotides of the center of the hybridization probe or at the center of said probe. In a preferred embodiment, the probes are selected from the group consisting of the sequences P1 to P125 and the complementary sequence thereto.

It should be noted that the polynucleotides of the present invention are not limited to having the exact flanking sequences surrounding the polymorphic bases which are enumerated in Sequence Listing. Rather, it will be appreciated that the flanking sequences surrounding the biallelic markers may be lengthened or shortened to any extent compatible with their intended use and the present invention specifically contemplates such sequences. The flanking regions outside of the contiguous span need not be homologous to native flanking sequences which actually occur in human subjects. The addition of any nucleotide sequence which is compatible with the nucleotides intended use is specifically contemplated.

Primers and probes may be labeled or immobilized on a solid support as described in " Oligonucleotide probes and primers".

The polynucleotides of the invention which are attached to a solid support encompass polynucleotides with any further limitation described in this disclosure, or those following, specified alone or in any combination: Optionally, said polynucleotides may be specified as attached individually or in groups of at least 2, 5, 8, 10, 12, 15, 20, or 25 distinct polynucleotides of the invention to a single solid support. Optionally, polynucleotides other than those of the invention may attached to the same solid support as polynucleotides of the invention. Optionally, when multiple polynucleotides are attached to a solid support they may be attached at random locations, or in an ordered array. Optionally, said ordered array may be addressable.

The present invention also encompasses diagnostic kits comprising one or more polynucleotides of the invention with a portion or all of the necessary reagents and instructions for genotyping a test subject by determining the identity of a nucleotide at a *PCTA-1*-related biallelic marker. The polynucleotides of a kit may optionally be attached to a solid support, or be part of an array or addressable array of polynucleotides. The kit may provide for the determination of the identity of the nucleotide at a marker position by any method known in the art including, but not limited to, a sequencing assay method, a microsequencing assay method, a hybridization assay method, or an enzyme-based mismatch detection assay method.

Methods For *De Novo* Identification Of Biallelic Markers

Any of a variety of methods can be used to screen a genomic fragment for single nucleotide polymorphisms such as differential hybridization with oligonucleotide probes, detection of changes in the mobility measured by gel electrophoresis or direct sequencing of the amplified nucleic acid. A preferred method for identifying biallelic markers involves comparative sequencing of genomic DNA fragments from an appropriate number of unrelated individuals.

In a first embodiment, DNA samples from unrelated individuals are pooled together, following which the genomic DNA of interest is amplified and sequenced. The nucleotide sequences thus obtained are then analyzed to identify significant polymorphisms. One of the major advantages of this method resides in the fact that the pooling of the DNA samples substantially reduces the number of DNA amplification reactions and sequencing reactions, which must be carried out. Moreover, this method is sufficiently sensitive so that a biallelic marker obtained thereby usually demonstrates a sufficient frequency of its less common allele to be useful in conducting association studies.

In a second embodiment, the DNA samples are not pooled and are therefore amplified and sequenced individually. This method is usually preferred when biallelic markers need to be identified in order to perform association studies within candidate genes. Preferably, highly relevant gene regions such as promoter regions or exon regions may be screened for biallelic markers. A biallelic marker obtained using this method may show a lower degree of informativeness for conducting association studies, e.g. if the frequency of its less frequent allele may be less than about 10%. Such a biallelic marker will, however, be sufficiently informative to conduct association studies and it will further be appreciated that including less informative biallelic markers in the genetic analysis studies of the present invention, may allow in some cases the direct identification of causal mutations, which may, depending on their penetrance, be rare mutations.

The following is a description of the various parameters of a preferred method used by the inventors for the identification of the biallelic markers of the present invention.

Genomic DNA Samples

The genomic DNA samples from which the biallelic markers of the present invention are generated are preferably obtained from unrelated individuals corresponding to a heterogeneous population of known ethnic background. The number of individuals from whom DNA samples are obtained can vary substantially, preferably from about 10 to about 1000, preferably from about 50 to about 200 individuals. It is usually preferred to collect DNA samples from at least about 100 individuals in order to have sufficient polymorphic diversity in a given population to identify as many markers as possible and to generate statistically significant results.

As for the source of the genomic DNA to be subjected to analysis, any test sample can be foreseen without any particular limitation. These test samples include biological samples, which can be tested by the methods of the present invention described herein, and include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine,

lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; fixed tissue specimens including tumor and non-tumor tissue and lymph node tissues; bone marrow aspirates and fixed cell specimens. The preferred source of genomic DNA used in the present invention is from peripheral venous blood of each donor. Techniques to prepare genomic DNA from biological samples are well known to the skilled technician. Details of a preferred embodiment are provided in Example 1. The person skilled in the art can choose to amplify pooled or unpooled DNA samples.

DNA Amplification

The identification of biallelic markers in a sample of genomic DNA may be facilitated through the use of DNA amplification methods. DNA samples can be pooled or unpooled for the amplification step. DNA amplification techniques are well known to those skilled in the art.

Amplification techniques that can be used in the context of the present invention include, but are not limited to, the ligase chain reaction (LCR) described in EP-A- 320 308, WO 9320227 and EP-A-439 182, the disclosures of which are incorporated herein by reference in their entireties, the polymerase chain reaction (PCR, RT-PCR) and techniques such as the nucleic acid sequence based amplification (NASBA) described in Guatelli J.C., et al.(1990) and in Compton J.(1991), Q-beta amplification as described in European Patent Application No 4544610, the disclosures of which are incorporated herein by reference in their entireties, strand displacement amplification as described in Walker et al.(1996) and EP A 684 315, the disclosures of which are incorporated herein by reference in their entireties, and, target mediated amplification as described in PCT Publication WO 9322461.

LCR and Gap LCR are exponential amplification techniques, both depend on DNA ligase to join adjacent primers annealed to a DNA molecule. In Ligase Chain Reaction (LCR), probe pairs are used which include two primary (first and second) and two secondary (third and fourth) probes, all of which are employed in molar excess to target. The first probe hybridizes to a first segment of the target strand and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so that the primary probes abut one another in 5' phosphate-3'hydroxyl relationship, and so that a ligase can covalently fuse or ligate the two probes into a fused product. In addition, a third (secondary) probe can hybridize to a portion of the first probe and a fourth (secondary) probe can hybridize to a portion of the second probe in a similar abutting fashion. Of course, if the target is initially double stranded, the secondary probes also will hybridize to the target complement in the first instance. Once the ligated strand of primary probes is separated from the target strand, it will hybridize with the third and fourth probes, which can be ligated to form a complementary, secondary ligated

product. It is important to realize that the ligated products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved. A method for multiplex LCR has also been described (WO 9320227, the disclosure of which is incorporated herein by reference in its entirety). Gap LCR (GLCR) is a version of LCR where the probes are not adjacent but are separated by 2 to 3 bases.

For amplification of mRNAs, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Patent No. 5,322,770, the disclosure of which is incorporated herein by reference in its entirety, or, to use Asymmetric Gap LCR (RT-AGLCR) as described by Marshall et al.(1994). AGLCR is a modification of GLCR that allows the amplification of RNA.

The PCR technology is the preferred amplification technique used in the present invention. A variety of PCR techniques are familiar to those skilled in the art. For a review of PCR technology, see White (1997) and the publication entitled "PCR Methods and Applications" (1991, Cold Spring Harbor Laboratory Press). In each of these PCR procedures, PCR primers on either side of the nucleic acid sequences to be amplified are added to a suitably prepared nucleic acid sample along with dNTPs and a thermostable polymerase such as Taq polymerase, Pfu polymerase, or Vent polymerase. The nucleic acid in the sample is denatured and the PCR primers are specifically hybridized to complementary nucleic acid sequences in the sample. The hybridized primers are extended. Thereafter, another cycle of denaturation, hybridization, and extension is initiated. The cycles are repeated multiple times to produce an amplified fragment containing the nucleic acid sequence between the primer sites. PCR has further been described in several patents including US Patents 4,683,195; 4,683,202; and 4,965,188, the disclosures of which are incorporated herein by reference in their entireties.

The PCR technology is the preferred amplification technique used to identify new biallelic markers. A typical example of a PCR reaction suitable for the purposes of the present invention is provided in Example 2.

One of the aspects of the present invention is a method for the amplification of the human *PCTA-1* gene, particularly of a fragment of the genomic sequence of SEQ ID No 1 or of the cDNA sequences of SEQ ID Nos 2, 3, 4, 8, or a fragment or a variant thereof in a test sample, preferably using the PCR technology. This method comprises the steps of:

a) contacting a test sample with amplification reaction reagents comprising a pair of amplification primers as described above and located on either side of the polynucleotide region to be amplified, and

b) optionally, detecting the amplification products.

The invention also concerns a kit for the amplification of a *PCTA-1* gene sequence, particularly of a portion of the genomic sequence of SEQ ID No 1 or of the cDNA sequences of SEQ ID Nos 2, 3 4, 9, or a variant thereof in a test sample, wherein said kit comprises:

a) a pair of oligonucleotide primers located on either side of the *PCTA-1* region to be amplified;

b) optionally, the reagents necessary for performing the amplification reaction.

In one embodiment of the above amplification method and kit, the amplification product is detected by hybridization with a labeled probe having a sequence which is complementary to the amplified region. In another embodiment of the above amplification method and kit, primers comprise a sequence which is selected from the group consisting of the nucleotide sequences of B1 to B47, C1 to C47, D1 to D125, and E1 to E125.

In a first embodiment of the present invention, biallelic markers are identified using genomic sequence information generated by the inventors. Sequenced genomic DNA fragments are used to design primers for the amplification of 500 bp fragments. These 500 bp fragments are amplified from genomic DNA and are scanned for biallelic markers. Primers may be designed using the OSP software (Hillier L. and Green P., 1991). All primers may contain, upstream of the specific target bases, a common oligonucleotide tail that serves as a sequencing primer. Those skilled in the art are familiar with primer extensions, which can be used for these purposes.

Preferred primers, useful for the amplification of genomic sequences encoding the candidate genes, focus on promoters, exons and splice sites of the genes. A biallelic marker presents a higher probability to be an eventual causal mutation if it is located in these functional regions of the gene. Preferred amplification primers of the invention include the nucleotide sequences B1 to B47 and C1 to C47, detailed further in Example 2, Table 1.

Sequencing Of Amplified Genomic DNA And Identification Of Single Nucleotide Polymorphisms

The amplification products generated as described above, are then sequenced using any method known and available to the skilled technician. Methods for sequencing DNA using either the dideoxy-mediated method (Sanger method) or the Maxam-Gilbert method are widely known to those of ordinary skill in the art. Such methods are for example disclosed in Sambrook et al.(1989). Alternative approaches include hybridization to high-density DNA probe arrays as described in Chee et al.(1996).

Preferably, the amplified DNA is subjected to automated dideoxy terminator sequencing reactions using a dye-primer cycle sequencing protocol. The products of the sequencing reactions are run on sequencing gels and the sequences are determined using gel image analysis. The polymorphism search is based on the presence of superimposed peaks in the electrophoresis pattern resulting from different bases occurring at the same position. Because each dideoxy terminator is labeled with a different fluorescent molecule, the two peaks corresponding to a biallelic site present distinct colors corresponding to two different nucleotides at the same position on the sequence. However, the presence of two peaks can be an artifact due to background noise. To exclude such an artifact, the two DNA strands are sequenced and a comparison between the peaks is carried out. In order to be registered as a polymorphic sequence, the polymorphism has to be detected on both strands.

The above procedure permits those amplification products, which contain biallelic markers to be identified. The detection limit for the frequency of biallelic polymorphisms detected by sequencing pools of 100 individuals is approximately 0.1 for the minor allele, as verified by sequencing pools of known allelic frequencies. However, more than 90% of the biallelic polymorphisms detected by the pooling method have a frequency for the minor allele higher than 0.25. Therefore, the biallelic markers selected by this method have a frequency of at least 0.1 for the minor allele and less than 0.9 for the major allele. Preferably at least 0.2 for the minor allele and less than 0.8 for the major allele, more preferably at least 0.3 for the minor allele and less than 0.7 for the major allele, thus a heterozygosity rate higher than 0.18, preferably higher than 0.32, more preferably higher than 0.42.

In another embodiment, biallelic markers are detected by sequencing individual DNA samples, the frequency of the minor allele of such a biallelic marker may be less than 0.1.

Validation Of The Biallelic Markers Of The Present Invention

The polymorphisms are evaluated for their usefulness as genetic markers by validating that both alleles are present in a population. Validation of the biallelic markers is accomplished by genotyping a group of individuals by a method of the invention and demonstrating that both alleles are present. Microsequencing is a preferred method of genotyping alleles. The validation by genotyping step may be performed on individual samples derived from each individual in the group or by genotyping a pooled sample derived from more than one individual. The group can be as small as one individual if that individual is heterozygous for the allele in question. Preferably the group contains at least three individuals, more preferably the group contains five or six individuals, so that a single validation test will be more likely to result in the validation of more of the biallelic markers that are being tested. It should be noted,

however, that when the validation test is performed on a small group it may result in a false negative result if as a result of sampling error none of the individuals tested carries one of the two alleles. Thus, the validation process is less useful in demonstrating that a particular initial result is an artifact, than it is at demonstrating that there is a *bona fide* biallelic marker at a particular position in a sequence. All of the genotyping, haplotyping, association, and interaction study methods of the invention may optionally be performed solely with validated biallelic markers.

Evaluation Of The Frequency Of The Biallelic Markers Of The Present Invention

The validated biallelic markers are further evaluated for their usefulness as genetic markers by determining the frequency of the least common allele at the biallelic marker site. The higher the frequency of the less common allele the greater the usefulness of the biallelic marker is association and interaction studies. The determination of the least common allele is accomplished by genotyping a group of individuals by a method of the invention and demonstrating that both alleles are present. This determination of frequency by genotyping step may be performed on individual samples derived from each individual in the group or by genotyping a pooled sample derived from more than one individual. The group must be large enough to be representative of the population as a whole. Preferably the group contains at least 20 individuals, more preferably the group contains at least 50 individuals, most preferably the group contains at least 100 individuals. Of course the larger the group the greater the accuracy of the frequency determination because of reduced sampling error. For an indication of the frequency for the less common allele of a particular biallelic marker of the invention see Table 2. A biallelic marker wherein the frequency of the less common allele is 30% or more is termed a "high quality biallelic marker." All of the genotyping, haplotyping, association, and interaction study methods of the invention may optionally be performed solely with high quality biallelic markers.

Methods For Genotyping An Individual For Biallelic Markers

Methods are provided to genotype a biological sample for one or more biallelic markers of the present invention, all of which may be performed *in vitro*. Such methods of genotyping comprise determining the identity of a nucleotide at a *PCTA-1* biallelic marker site by any method known in the art. These methods find use in genotyping case-control populations in association studies as well as individuals in the context of detection of alleles of biallelic markers which are known to be associated with a given trait, in which case both copies of the biallelic marker present in individual's genome are determined so that an individual may be classified as homozygous or heterozygous for a particular allele.

These genotyping methods can be performed on nucleic acid samples derived from a single individual or pooled DNA samples.

Genotyping can be performed using similar methods as those described above for the identification of the biallelic markers, or using other genotyping methods such as those further described below. In preferred embodiments, the comparison of sequences of amplified genomic fragments from different individuals is used to identify new biallelic markers whereas microsequencing is used for genotyping known biallelic markers in diagnostic and association study applications.

In one embodiment the invention encompasses methods of genotyping comprising determining the identity of a nucleotide at a *PCTA-1*-related biallelic marker or the complement thereof in a biological sample; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said biological sample is derived from a single subject; optionally, wherein the identity of the nucleotides at said biallelic marker is determined for both copies of said biallelic marker present in said individual's genome; optionally, wherein said biological sample is derived from multiple subjects; Optionally, the genotyping methods of the invention encompass methods with any further limitation described in this disclosure, or those following, specified alone or in any combination; Optionally, said method is performed *in vitro*; optionally, further comprising amplifying a portion of said sequence comprising the biallelic marker prior to said determining step; Optionally, wherein said amplifying is performed by PCR, LCR, or replication of a recombinant vector comprising an origin of replication and said fragment in a host cell; optionally, wherein said determining is performed by a hybridization assay, a sequencing assay, a microsequencing assay, or an enzyme-based mismatch detection assay.

Source of Nucleic Acids for genotyping

Any source of nucleic acids, in purified or non-purified form, can be utilized as the starting nucleic acid, provided it contains or is suspected of containing the specific nucleic acid sequence desired. DNA or RNA may be extracted from cells, tissues, body fluids and the like as described above. While nucleic acids for use in the genotyping methods of the invention can be derived from any mammalian source, the test subjects and individuals from which nucleic acid samples are taken are generally understood to be human.

Amplification Of DNA Fragments Comprising Biallelic Markers

Methods and polynucleotides are provided to amplify a segment of nucleotides comprising one or more biallelic marker of the present invention. It will be appreciated that amplification of DNA fragments comprising biallelic markers may be used in various methods and for various purposes and is not restricted to genotyping. Nevertheless, many genotyping methods, although not all, require the previous amplification of the DNA region carrying the biallelic marker of interest. Such methods specifically increase the concentration or total number of sequences that span the biallelic marker or include that site and sequences located either distal or proximal to it. Diagnostic assays may also rely on amplification of DNA segments carrying a biallelic marker of the present invention. Amplification of DNA may be achieved by any method known in the art. Amplification techniques are described above in the section entitled, "DNA amplification."

The invention also concerns a method for the amplification of a *PCTA-1* gene region, preferably containing at least one of the polymorphic bases identified in the context of the present invention, or a fragment or variant thereof, in a test sample. The method comprises the step of contacting a test sample suspected of containing the targeted *PCTA-1* gene sequence or a fragment thereof with amplification reaction reagents comprising a pair of amplification primers, preferably located on either side of the polymorphic base. Preferred amplification primers consist of B1 to B47 and C1 to C47. The method may further comprise the step of detecting the amplification product. For example, the amplification product may be detected using a detection probe that can hybridize with an internal region of the amplicon sequences. In some embodiments, the polymorphic base is included in one of the sequences of P1 to P125, and the complementary sequences thereof.

Some of these amplification methods are particularly suited for the detection of single nucleotide polymorphisms and allow the simultaneous amplification of a target sequence and the identification of the polymorphic nucleotide as it is further described below.

The identification of biallelic markers as described above allows the design of appropriate oligonucleotides, which can be used as primers to amplify DNA fragments comprising the biallelic markers of the present invention. Amplification can be performed using the primers initially used to discover new biallelic markers which are described herein or any set of primers allowing the amplification of a DNA fragment comprising a biallelic marker of the present invention.

In some embodiments the present invention provides primers for amplifying a DNA fragment containing one or more biallelic markers of the present invention. Preferred amplification primers are listed in Example 2. It will be appreciated that the primers listed are merely exemplary and that any other set of primers which produce amplification products containing one or more biallelic markers of the present invention are also of use.

The spacing of the primers determines the length of the segment to be amplified. In the context of the present invention, amplified segments carrying biallelic markers can range in size from at least about 25 bp to 35 kbp. Amplification fragments from 25-3000 bp are typical, fragments from 50-1000 bp are preferred and fragments from 100-600 bp are highly preferred. It will be appreciated that amplification primers for the biallelic markers may be any sequence which allow the specific amplification of any DNA fragment carrying the markers. Amplification primers may be labeled or immobilized on a solid support as described in "Oligonucleotide probes and primers".

Methods of Genotyping DNA samples for Biallelic Markers

Any method known in the art can be used to identify the nucleotide present at a biallelic marker site. Since the biallelic marker allele to be detected has been identified and specified in the present invention, detection will prove simple for one of ordinary skill in the art by employing any of a number of techniques. Many genotyping methods require the previous amplification of the DNA region carrying the biallelic marker of interest. While the amplification of target or signal is often preferred at present, ultrasensitive detection methods which do not require amplification are also encompassed by the present genotyping methods. Methods well-known to those skilled in the art that can be used to detect biallelic polymorphisms include methods such as, conventional dot blot analyzes, single strand conformational polymorphism analysis (SSCP) described by Orita et al.(1989), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, and other conventional techniques as described in Sheffield et al.(1991), White et al.(1992), Grompe et al.(1989 and 1993). Another method for determining the identity of the nucleotide present at

a particular polymorphic site employs a specialized exonuclease-resistant nucleotide derivative as described in US patent 4,656,127.

Preferred methods involve directly determining the identity of the nucleotide present at a biallelic marker site by sequencing assay, enzyme-based mismatch detection assay, or hybridization assay. The following is a description of some preferred methods. A highly preferred method is the microsequencing technique. The term "sequencing" is generally used herein to refer to polymerase extension of duplex primer/template complexes and includes both traditional sequencing and microsequencing.

1) Sequencing Assays

The nucleotide present at a polymorphic site can be determined by sequencing methods. In a preferred embodiment, DNA samples are subjected to PCR amplification before sequencing as described above. DNA sequencing methods are described in "Sequencing Of Amplified Genomic DNA And Identification Of Single Nucleotide Polymorphisms".

Preferably, the amplified DNA is subjected to automated dideoxy terminator sequencing reactions using a dye-primer cycle sequencing protocol. Sequence analysis allows the identification of the base present at the biallelic marker site.

2) Microsequencing Assays

In microsequencing methods, the nucleotide at a polymorphic site in a target DNA is detected by a single nucleotide primer extension reaction. This method involves appropriate microsequencing primers which, hybridize just upstream of the polymorphic base of interest in the target nucleic acid. A polymerase is used to specifically extend the 3' end of the primer with one single ddNTP (chain terminator) complementary to the nucleotide at the polymorphic site. Next the identity of the incorporated nucleotide is determined in any suitable way.

Typically, microsequencing reactions are carried out using fluorescent ddNTPs and the extended microsequencing primers are analyzed by electrophoresis on ABI 377 sequencing machines to determine the identity of the incorporated nucleotide as described in EP 412 883, the disclosure of which is incorporated herein by reference in its entirety. Alternatively capillary electrophoresis can be used in order to process a higher number of assays simultaneously. An example of a typical microsequencing procedure that can be used in the context of the present invention is provided in Example 4.

Different approaches can be used for the labeling and detection of ddNTPs. A homogeneous phase detection method based on fluorescence resonance energy transfer has been described by Chen and Kwok (1997) and Chen et al.(1997). In this method, amplified genomic DNA fragments containing polymorphic sites are incubated with a 5'-fluorescein–labeled

primer in the presence of allelic dye-labeled dideoxyribonucleoside triphosphates and a modified Taq polymerase. The dye-labeled primer is extended one base by the dye-terminator specific for the allele present on the template. At the end of the genotyping reaction, the fluorescence intensities of the two dyes in the reaction mixture are analyzed directly without separation or purification. All these steps can be performed in the same tube and the fluorescence changes can be monitored in real time. Alternatively, the extended primer may be analyzed by MALDI-TOF Mass Spectrometry. The base at the polymorphic site is identified by the mass added onto the microsequencing primer (see Haff and Smirnov, 1997).

Microsequencing may be achieved by the established microsequencing method or by developments or derivatives thereof. Alternative methods include several solid-phase microsequencing techniques. The basic microsequencing protocol is the same as described previously, except that the method is conducted as a heterogeneous phase assay, in which the primer or the target molecule is immobilized or captured onto a solid support. To simplify the primer separation and the terminal nucleotide addition analysis, oligonucleotides are attached to solid supports or are modified in such ways that permit affinity separation as well as polymerase extension. The 5' ends and internal nucleotides of synthetic oligonucleotides can be modified in a number of different ways to permit different affinity separation approaches, e.g., biotinylation. If a single affinity group is used on the oligonucleotides, the oligonucleotides can be separated from the incorporated terminator regent. This eliminates the need of physical or size separation. More than one oligonucleotide can be separated from the terminator reagent and analyzed simultaneously if more than one affinity group is used. This permits the analysis of several nucleic acid species or more nucleic acid sequence information per extension reaction. The affinity group need not be on the priming oligonucleotide but could alternatively be present on the template. For example, immobilization can be carried out via an interaction between biotinylated DNA and streptavidin-coated microtitration wells or avidin-coated polystyrene particles. In the same manner, oligonucleotides or templates may be attached to a solid support in a high-density format. In such solid phase microsequencing reactions, incorporated ddNTPs can be radiolabeled (Syvänen, 1994) or linked to fluorescein (Livak and Hainer, 1994). The detection of radiolabeled ddNTPs can be achieved through scintillation-based techniques. The detection of fluorescein-linked ddNTPs can be based on the binding of antifluorescein antibody conjugated with alkaline phosphatase, followed by incubation with a chromogenic substrate (such as *p*-nitrophenyl phosphate). Other possible reporter-detection pairs include: ddNTP linked to dinitrophenyl (DNP) and anti-DNP alkaline phosphatase conjugate (Harju et al., 1993) or biotinylated ddNTP and horseradish peroxidase-conjugated streptavidin with *o*-phenylenediamine as a substrate (WO 92/15712, the disclosure of which is

incorporated herein by reference in its entirety). As yet another alternative solid-phase microsequencing procedure, Nyren et al.(1993) described a method relying on the detection of DNA polymerase activity by an enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA).

Pastinen et al.(1997) describe a method for multiplex detection of single nucleotide polymorphism in which the solid phase minisequencing principle is applied to an oligonucleotide array format. High-density arrays of DNA probes attached to a solid support (DNA chips) are further described below.

In one aspect the present invention provides polynucleotides and methods to genotype one or more biallelic markers of the present invention by performing a microsequencing assay. Preferred microsequencing primers include the nucleotide sequences D1 to D125 and E1 to E125. More preferred microsequencing primers are selected from the group consisting of the nucleotide sequences D15, D24, D30, D34, D36, D38, D41, D44, D50, D53, D54, D56, D57, D59, D76, D85, D93, D108, D111, D115, D124, E11, E14, E22, E25, E26, E35, E42, E52, E53, E55, E56, E60, E61, E64, E73, E75, E93, E96. It will be appreciated that the microsequencing primers listed in Example 4 are merely exemplary and that, any primer having a 3' end immediately adjacent to the polymorphic nucleotide may be used. Similarly, it will be appreciated that microsequencing analysis may be performed for any biallelic marker or any combination of biallelic markers of the present invention. One aspect of the present invention is a solid support which includes one or more microsequencing primers listed in Example 4, or fragments comprising at least 8, 12, 15, 20, 25, 30, 40, or 50 consecutive nucleotides thereof, to the extent that such lengths are consistent with the primer described, and having a 3' terminus immediately upstream of the corresponding biallelic marker, for determining the identity of a nucleotide at a biallelic marker site.

3) Mismatch detection assays based on polymerases and ligases

In one aspect the present invention provides polynucleotides and methods to determine the allele of one or more biallelic markers of the present invention in a biological sample, by mismatch detection assays based on polymerases and/or ligases. These assays are based on the specificity of polymerases and ligases. Polymerization reactions places particularly stringent requirements on correct base pairing of the 3' end of the amplification primer and the joining of two oligonucleotides hybridized to a target DNA sequence is quite sensitive to mismatches close to the ligation site, especially at the 3' end. Methods, primers and various parameters to amplify DNA fragments comprising biallelic markers of the present invention are further described above in "Amplification Of DNA Fragments Comprising Biallelic Markers".

Allele Specific Amplification Primers

Discrimination between the two alleles of a biallelic marker can also be achieved by allele specific amplification, a selective strategy, whereby one of the alleles is amplified without amplification of the other allele. For allele specific amplification, at least one member of the pair of primers is sufficiently complementary with a region of a *PCTA-1* gene comprising the polymorphic base of a biallelic marker of the present invention to hybridize therewith and to initiate the amplification. Such primers are able to discriminate between the two alleles of a biallelic marker.

This is accomplished by placing the polymorphic base at the 3' end of one of the amplification primers. Because the extension forms from the 3'end of the primer, a mismatch at or near this position has an inhibitory effect on amplification. Therefore, under appropriate amplification conditions, these primers only direct amplification on their complementary allele. Determining the precise location of the mismatch and the corresponding assay conditions are well within the ordinary skill in the art.

Ligation/Amplification Based Methods

The "Oligonucleotide Ligation Assay" (OLA) uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target molecules. One of the oligonucleotides is biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate that can be captured and detected. OLA is capable of detecting single nucleotide polymorphisms and may be advantageously combined with PCR as described by Nickerson et al.(1990). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

Other amplification methods which are particularly suited for the detection of single nucleotide polymorphism include LCR (ligase chain reaction), Gap LCR (GLCR) which are described above in "DNA Amplification". LCR uses two pairs of probes to exponentially amplify a specific target. The sequences of each pair of oligonucleotides, is selected to permit the pair to hybridize to abutting sequences of the same strand of the target. Such hybridization forms a substrate for a template-dependant ligase. In accordance with the present invention, LCR can be performed with oligonucleotides having the proximal and distal sequences of the same strand of a biallelic marker site. In one embodiment, either oligonucleotide will be designed to include the biallelic marker site. In such an embodiment, the reaction conditions are selected such that the oligonucleotides can be ligated together only if the target molecule either contains or lacks the specific nucleotide that is complementary to the biallelic marker on

the oligonucleotide. In an alternative embodiment, the oligonucleotides will not include the biallelic marker, such that when they hybridize to the target molecule, a "gap" is created as described in WO 90/01069, the disclosure of which is incorporated herein by reference in its entirety. This gap is then "filled" with complementary dNTPs (as mediated by DNA polymerase), or by an additional pair of oligonucleotides. Thus at the end of each cycle, each single strand has a complement capable of serving as a target during the next cycle and exponential allele-specific amplification of the desired sequence is obtained.

Ligase/Polymerase-mediated Genetic Bit Analysis™ is another method for determining the identity of a nucleotide at a preselected site in a nucleic acid molecule (WO 95/21271, the disclosure of which is incorporated herein by reference in its entirety). This method involves the incorporation of a nucleoside triphosphate that is complementary to the nucleotide present at the preselected site onto the terminus of a primer molecule, and their subsequent ligation to a second oligonucleotide. The reaction is monitored by detecting a specific label attached to the reaction's solid phase or by detection in solution.

4) Hybridization Assay Methods

A preferred method of determining the identity of the nucleotide present at a biallelic marker site involves nucleic acid hybridization. The hybridization probes, which can be conveniently used in such reactions, preferably include the probes defined herein. Any hybridization assay may be used including Southern hybridization, Northern hybridization, dot blot hybridization and solid-phase hybridization (see Sambrook et al., 1989).

Hybridization refers to the formation of a duplex structure by two single stranded nucleic acids due to complementary base pairing. Hybridization can occur between exactly complementary nucleic acid strands or between nucleic acid strands that contain minor regions of mismatch. Specific probes can be designed that hybridize to one form of a biallelic marker and not to the other and therefore are able to discriminate between different allelic forms. Allele-specific probes are often used in pairs, one member of a pair showing perfect match to a target sequence containing the original allele and the other showing a perfect match to the target sequence containing the alternative allele. Hybridization conditions should be sufficiently stringent that there is a significant difference in hybridization intensity between alleles, and preferably an essentially binary response, whereby a probe hybridizes to only one of the alleles. Stringent, sequence specific hybridization conditions, under which a probe will hybridize only to the exactly complementary target sequence are well known in the art (Sambrook et al., 1989). Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting

point (Tm) for the specific sequence at a defined ionic strength and pH. Although such hybridization can be performed in solution, it is preferred to employ a solid-phase hybridization assay. The target DNA comprising a biallelic marker of the present invention may be amplified prior to the hybridization reaction. The presence of a specific allele in the sample is determined by detecting the presence or the absence of stable hybrid duplexes formed between the probe and the target DNA. The detection of hybrid duplexes can be carried out by a number of methods. Various detection assay formats are well known which utilize detectable labels bound to either the target or the probe to enable detection of the hybrid duplexes. Typically, hybridization duplexes are separated from unhybridized nucleic acids and the labels bound to the duplexes are then detected. Those skilled in the art will recognize that wash steps may be employed to wash away excess target DNA or probe as well as unbound conjugate. Further, standard heterogeneous assay formats are suitable for detecting the hybrids using the labels present on the primers and probes.

Two recently developed assays allow hybridization-based allele discrimination with no need for separations or washes (see Landegren U. et al., 1998). The TaqMan assay takes advantage of the 5' nuclease activity of Taq DNA polymerase to digest a DNA probe annealed specifically to the accumulating amplification product. TaqMan probes are labeled with a donor-acceptor dye pair that interacts via fluorescence energy transfer. Cleavage of the TaqMan probe by the advancing polymerase during amplification dissociates the donor dye from the quenching acceptor dye, greatly increasing the donor fluorescence. All reagents necessary to detect two allelic variants can be assembled at the beginning of the reaction and the results are monitored in real time (see Livak et al., 1995). In an alternative homogeneous hybridization based procedure, molecular beacons are used for allele discriminations. Molecular beacons are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acids in homogeneous solutions. When they bind to their targets they undergo a conformational reorganization that restores the fluorescence of an internally quenched fluorophore (Tyagi et al., 1998).

The polynucleotides provided herein can be used to produce probes which can be used in hybridization assays for the detection of biallelic marker alleles in biological samples. These probes are characterized in that they preferably comprise between 8 and 50 nucleotides, and in that they are sufficiently complementary to a sequence comprising a biallelic marker of the present invention to hybridize thereto and preferably sufficiently specific to be able to discriminate the targeted sequence for only one nucleotide variation. A particularly preferred probe is 25 nucleotides in length. Another particularly preferred probe is 47 nucleotides in length. Preferably the biallelic marker is within 4 nucleotides of the center of the

polynucleotide probe. In particularly preferred probes, the biallelic marker is at the center of said polynucleotide. Preferred probes comprise a nucleotide sequence selected from the group consisting of amplicons listed in Table 1 and the sequences complementary thereto, or a fragment thereof, said fragment comprising at least about 8 consecutive nucleotides, preferably 10, 15, 20, more preferably 25, 30, 40, 47, or 50 consecutive nucleotides and containing a polymorphic base. Preferred probes comprise a nucleotide sequence selected from the group consisting of P1 to P125 and the sequences complementary thereto. In preferred embodiments the polymorphic base(s) are within 5, 4, 3, 2, 1, nucleotides of the center of the said polynucleotide, more preferably at the center of said polynucleotide.

Preferably the probes of the present invention are labeled or immobilized on a solid support. Labels and solid supports are further described in "Oligonucleotide Probes and Primers". The probes can be non-extendable as described in "Oligonucleotide Probes and Primers".

By assaying the hybridization to an allele specific probe, one can detect the presence or absence of a biallelic marker allele in a given sample. High-Throughput parallel hybridization in array format is specifically encompassed within "hybridization assays" and are described below.

5) Hybridization To Addressable Arrays Of Oligonucleotides

Hybridization assays based on oligonucleotide arrays rely on the differences in hybridization stability of short oligonucleotides to perfectly matched and mismatched target sequence variants. Efficient access to polymorphism information is obtained through a basic structure comprising high-density arrays of oligonucleotide probes attached to a solid support (e.g., the chip) at selected positions. Each DNA chip can contain thousands to millions of individual synthetic DNA probes arranged in a grid-like pattern and miniaturized to the size of a dime.

The chip technology has already been applied with success in numerous cases. For example, the screening of mutations has been undertaken in the BRCA1 gene, in *S. cerevisiae* mutant strains, and in the protease gene of HIV-1 virus (Hacia et al., 1996; Shoemaker et al., 1996; Kozal et al., 1996). Chips of various formats for use in detecting biallelic polymorphisms can be produced on a customized basis by Affymetrix (GeneChip™), Hyseq (HyChip and HyGnostics), and Protogene Laboratories.

In general, these methods employ arrays of oligonucleotide probes that are complementary to target nucleic acid sequence segments from an individual which, target sequences include a polymorphic marker. EP 785280, the disclosure of which is incorporated

herein by reference in its entirety, describes a tiling strategy for the detection of single nucleotide polymorphisms. Briefly, arrays may generally be "tiled" for a large number of specific polymorphisms. By "tiling" is generally meant the synthesis of a defined set of oligonucleotide probes which is made up of a sequence complementary to the target sequence of interest, as well as preselected variations of that sequence, e.g., substitution of one or more given positions with one or more members of the basis set of nucleotides. Tiling strategies are further described in PCT application No. WO 95/11995, the disclosure of which is incorporated herein by reference in its entirety. In a particular aspect, arrays are tiled for a number of specific, identified biallelic marker sequences. In particular, the array is tiled to include a number of detection blocks, each detection block being specific for a specific biallelic marker or a set of biallelic markers. For example, a detection block may be tiled to include a number of probes, which span the sequence segment that includes a specific polymorphism. To ensure probes that are complementary to each allele, the probes are synthesized in pairs differing at the biallelic marker. In addition to the probes differing at the polymorphic base, monosubstituted probes are also generally tiled within the detection block. These monosubstituted probes have bases at and up to a certain number of bases in either direction from the polymorphism, substituted with the remaining nucleotides (selected from A, T, G, C and U). Typically the probes in a tiled detection block will include substitutions of the sequence positions up to and including those that are 5 bases away from the biallelic marker. The monosubstituted probes provide internal controls for the tiled array, to distinguish actual hybridization from artefactual cross-hybridization. Upon completion of hybridization with the target sequence and washing of the array, the array is scanned to determine the position on the array to which the target sequence hybridizes. The hybridization data from the scanned array is then analyzed to identify which allele or alleles of the biallelic marker are present in the sample. Hybridization and scanning may be carried out as described in PCT application No. WO 92/10092 and WO 95/11995 and US patent No. 5,424,186, the disclosures of which are incorporated herein by reference in their entireties.

Thus, in some embodiments, the chips may comprise an array of nucleic acid sequences of fragments of about 15 nucleotides in length. In further embodiments, the chip may comprise an array including at least one of the sequences selected from the group consisting of amplicons listed in table 1 and the sequences complementary thereto, or a fragment thereof, said fragment comprising at least about 8 consecutive nucleotides, preferably 10, 15, 20, more preferably 25, 30, 40, 47, or 50 consecutive nucleotides and containing a polymorphic base. In preferred embodiments the polymorphic base is within 5, 4, 3, 2, 1, nucleotides of the center of the said polynucleotide, more preferably at the center of said polynucleotide. In some embodiments, the

chip may comprise an array of at least 2, 3, 4, 5, 6, 7, 8 or more of these polynucleotides of the invention. Solid supports and polynucleotides of the present invention attached to solid supports are further described in "Oligonucleotide Probes And Primers".

6) Integrated Systems

Another technique, which may be used to analyze polymorphisms, includes multicomponent integrated systems, which miniaturize and compartmentalize processes such as PCR and capillary electrophoresis reactions in a single functional device. An example of such technique is disclosed in US patent 5,589,136, the disclosure of which is incorporated herein by reference in its entirety, which describes the integration of PCR amplification and capillary electrophoresis in chips.

Integrated systems can be envisaged mainly when microfluidic systems are used. These systems comprise a pattern of microchannels designed onto a glass, silicon, quartz, or plastic wafer included on a microchip. The movements of the samples are controlled by electric, electroosmotic or hydrostatic forces applied across different areas of the microchip to create functional microscopic valves and pumps with no moving parts.

For genotyping biallelic markers, the microfluidic system may integrate nucleic acid amplification, microsequencing, capillary electrophoresis and a detection method such as laser-induced fluorescence detection.

Methods Of Genetic Analysis Using The Biallelic Markers Of The Present Invention

Different methods are available for the genetic analysis of complex traits (see Lander and Schork, 1994). The search for disease-susceptibility genes is conducted using two main methods: the linkage approach in which evidence is sought for cosegregation between a locus and a putative trait locus using family studies, and the association approach in which evidence is sought for a statistically significant association between an allele or a trait causing allele and a trait (Khoury et al., 1993). In general, the biallelic markers of the present invention find use in any method known in the art to demonstrate a statistically significant correlation between a genotype and a phenotype. The biallelic markers may be used in parametric and non-parametric linkage analysis methods. Preferably, the biallelic markers of the present invention are used to identify genes associated with detectable traits using association studies, an approach which does not require the use of affected families and which permits the identification of genes associated with complex and sporadic traits.

The genetic analysis using the biallelic markers of the present invention may be conducted on any scale. The whole set of biallelic markers of the present invention or any subset of biallelic markers of the present invention corresponding to the candidate gene may be

used. Further, any set of genetic markers including a biallelic marker of the present invention may be used. A set of biallelic polymorphisms that could be used as genetic markers in combination with the biallelic markers of the present invention has been described in WO 98/20165, the disclosure of which is incorporated herein by reference in its entirety. As mentioned above, it should be noted that the biallelic markers of the present invention may be included in any complete or partial genetic map of the human genome. These different uses are specifically contemplated in the present invention and claims.

Linkage Analysis

Linkage analysis is based upon establishing a correlation between the transmission of genetic markers and that of a specific trait throughout generations within a family. Thus, the aim of linkage analysis is to detect marker loci that show cosegregation with a trait of interest in pedigrees.

Parametric Methods

When data are available from successive generations there is the opportunity to study the degree of linkage between pairs of loci. Estimates of the recombination fraction enable loci to be ordered and placed onto a genetic map. With loci that are genetic markers, a genetic map can be established, and then the strength of linkage between markers and traits can be calculated and used to indicate the relative positions of markers and genes affecting those traits (Weir, 1996). The classical method for linkage analysis is the logarithm of odds (lod) score method (see Morton, 1955; Ott, 1991). Calculation of lod scores requires specification of the mode of inheritance for the disease (parametric method). Generally, the length of the candidate region identified using linkage analysis is between 2 and 20Mb. Once a candidate region is identified as described above, analysis of recombinant individuals using additional markers allows further delineation of the candidate region. Linkage analysis studies have generally relied on the use of a maximum of 5,000 microsatellite markers, thus limiting the maximum theoretical attainable resolution of linkage analysis to about 600 kb on average.

Linkage analysis has been successfully applied to map simple genetic traits that show clear Mendelian inheritance patterns and which have a high penetrance (i.e., the ratio between the number of trait positive carriers of allele *a* and the total number of *a* carriers in the population). However, parametric linkage analysis suffers from a variety of drawbacks. First, it is limited by its reliance on the choice of a genetic model suitable for each studied trait. Furthermore, as already mentioned, the resolution attainable using linkage analysis is limited, and complementary studies are required to refine the analysis of the typical 2Mb to 20Mb regions initially identified through linkage analysis. In addition, parametric linkage analysis

approaches have proven difficult when applied to complex genetic traits, such as those due to the combined action of multiple genes and/or environmental factors. It is very difficult to model these factors adequately in a lod score analysis. In such cases, too large an effort and cost are needed to recruit the adequate number of affected families required for applying linkage analysis to these situations, as recently discussed by Risch, N. and Merikangas, K. (1996).

Non-Parametric Methods

The advantage of the so-called non-parametric methods for linkage analysis is that they do not require specification of the mode of inheritance for the disease, they tend to be more useful for the analysis of complex traits. In non-parametric methods, one tries to prove that the inheritance pattern of a chromosomal region is not consistent with random Mendelian segregation by showing that affected relatives inherit identical copies of the region more often than expected by chance. Affected relatives should show excess "allele sharing" even in the presence of incomplete penetrance and polygenic inheritance. In non-parametric linkage analysis the degree of agreement at a marker locus in two individuals can be measured either by the number of alleles identical by state (IBS) or by the number of alleles identical by descent (IBD). Affected sib pair analysis is a well-known special case and is the simplest form of these methods.

The biallelic markers of the present invention may be used in both parametric and non-parametric linkage analysis. Preferably biallelic markers may be used in non-parametric methods which allow the mapping of genes involved in complex traits. The biallelic markers of the present invention may be used in both IBD- and IBS- methods to map genes affecting a complex trait. In such studies, taking advantage of the high density of biallelic markers, several adjacent biallelic marker loci may be pooled to achieve the efficiency attained by multi-allelic markers (Zhao et al., 1998).

Population Association Studies

The present invention comprises methods for identifying if the *PCTA-1* gene is associated with a detectable trait using the biallelic markers of the present invention. In one embodiment the present invention comprises methods to detect an association between a biallelic marker allele or a biallelic marker haplotype and a trait. Further, the invention comprises methods to identify a trait causing allele in linkage disequilibrium with any biallelic marker allele of the present invention.

Alternative approaches can be employed to perform association studies: genome-wide association studies, candidate region association studies and candidate gene association studies. In a preferred embodiment, the biallelic markers of the present invention are used to perform

candidate gene association studies. The candidate gene analysis clearly provides a short-cut approach to the identification of genes and gene polymorphisms related to a particular trait when some information concerning the biology of the trait is available. Further, the biallelic markers of the present invention may be incorporated in any map of genetic markers of the human genome in order to perform genome-wide association studies. Methods to generate a high-density map of biallelic markers has been described in US Provisional Patent application serial number 60/082,614. The biallelic markers of the present invention may further be incorporated in any map of a specific candidate region of the genome (a specific chromosome or a specific chromosomal segment for example).

As mentioned above, association studies may be conducted within the general population and are not limited to studies performed on related individuals in affected families. Association studies are extremely valuable as they permit the analysis of sporadic or multifactor traits. Moreover, association studies represent a powerful method for fine-scale mapping enabling much finer mapping of trait causing alleles than linkage studies. Studies based on pedigrees often only narrow the location of the trait causing allele. Association studies using the biallelic markers of the present invention can therefore be used to refine the location of a trait causing allele in a candidate region identified by Linkage Analysis methods. Moreover, once a chromosome segment of interest has been identified, the presence of a candidate gene such as a candidate gene of the present invention, in the region of interest can provide a shortcut to the identification of the trait causing allele. Biallelic markers of the present invention can be used to demonstrate that a candidate gene is associated with a trait. Such uses are specifically contemplated in the present invention.

Determining The Frequency Of A Biallelic Marker Allele Or Of A Biallelic Marker Haplotype In A Population

Association studies explore the relationships among frequencies for sets of alleles between loci.

Determining The Frequency Of An Allele In A Population

Allelic frequencies of the biallelic markers in a populations can be determined using one of the methods described above under the heading "Methods For Genotyping An Individual For Biallelic Markers", or any genotyping procedure suitable for this intended purpose. Genotyping pooled samples or individual samples can determine the frequency of a biallelic marker allele in a population. One way to reduce the number of genotypings required is to use pooled samples. A major obstacle in using pooled samples is in terms of accuracy and reproducibility for determining accurate DNA concentrations in setting up the pools. Genotyping individual

samples provides higher sensitivity, reproducibility and accuracy and; is the preferred method used in the present invention. Preferably, each individual is genotyped separately and simple gene counting is applied to determine the frequency of an allele of a biallelic marker or of a genotype in a given population.

The invention also relates to methods of estimating the frequency of a *PCTA-1*-related biallelic marker allele in a population comprising: a) genotyping individuals from said population for said biallelic marker according to the method of the present invention; and b) determining the proportional representation of said biallelic marker in said population. In addition, the methods of estimating the frequency of an allele in a population of the invention encompass methods with any further limitation described in this disclosure, or those following, specified alone or in any combination; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, determining the frequency of a biallelic marker allele in a population may be accomplished by determining the identity of the nucleotides for both copies of said biallelic marker present in the genome of each individual in said population and calculating the proportional representation of said nucleotide at said *PCTA-1*-related biallelic marker for the population; Optionally, determining the proportional representation may be accomplished by performing a genotyping method of the invention on a pooled biological sample derived from a representative number of individuals, or each individual, in said population, and calculating the proportional amount of said nucleotide compared with the total.

Determining The Frequency Of A Haplotype In A Population

The gametic phase of haplotypes is unknown when diploid individuals are heterozygous at more than one locus. Using genealogical information in families gametic phase can

sometimes be inferred (Perlin et al., 1994). When no genealogical information is available different strategies may be used. One possibility is that the multiple-site heterozygous diploids can be eliminated from the analysis, keeping only the homozygotes and the single-site heterozygote individuals, but this approach might lead to a possible bias in the sample composition and the underestimation of low-frequency haplotypes. Another possibility is that single chromosomes can be studied independently, for example, by asymmetric PCR amplification (see Newton et al, 1989; Wu et al., 1989) or by isolation of single chromosome by limit dilution followed by PCR amplification (see Ruano et al., 1990). Further, a sample may be haplotyped for sufficiently close biallelic markers by double PCR amplification of specific alleles (Sarkar, G. and Sommer S. S., 1991). These approaches are not entirely satisfying either because of their technical complexity, the additional cost they entail, their lack of generalization at a large scale, or the possible biases they introduce. To overcome these difficulties, an algorithm to infer the phase of PCR-amplified DNA genotypes introduced by Clark, A.G.(1990) may be used. Briefly, the principle is to start filling a preliminary list of haplotypes present in the sample by examining unambiguous individuals, that is, the complete homozygotes and the single-site heterozygotes. Then other individuals in the same sample are screened for the possible occurrence of previously recognized haplotypes. For each positive identification, the complementary haplotype is added to the list of recognized haplotypes, until the phase information for all individuals is either resolved or identified as unresolved. This method assigns a single haplotype to each multiheterozygous individual, whereas several haplotypes are possible when there are more than one heterozygous site. Alternatively, one can use methods estimating haplotype frequencies in a population without assigning haplotypes to each individual. Preferably, a method based on an expectation-maximization (EM) algorithm (Dempster et al., 1977) leading to maximum-likelihood estimates of haplotype frequencies under the assumption of Hardy-Weinberg proportions (random mating) is used (see Excoffier L. and Slatkin M., 1995). The EM algorithm is a generalized iterative maximum-likelihood approach to estimation that is useful when data are ambiguous and/or incomplete. The EM algorithm is used to resolve heterozygotes into haplotypes. Haplotype estimations are further described below under the heading "Statistical Methods." Any other method known in the art to determine or to estimate the frequency of a haplotype in a population may be used.

The invention also encompasses methods of estimating the frequency of a haplotype for a set of biallelic markers in a population, comprising the steps of: a) genotyping at least one *PCTA-1*-related biallelic marker according to a method of the invention for each individual in said population; b) genotyping a second biallelic marker by determining the identity of the nucleotides at said second biallelic marker for both copies of said second biallelic marker

present in the genome of each individual in said population; and c) applying a haplotype determination method to the identities of the nucleotides determined in steps a) and b) to obtain an estimate of said frequency. In addition, the methods of estimating the frequency of a haplotype of the invention encompass methods with any further limitation described in this disclosure, or those following, specified alone or in any combination: optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, said haplotype determination method is performed by asymmetric PCR amplification, double PCR amplification of specific alleles, the Clark algorithm, or an expectation-maximization algorithm.

Linkage Disequilibrium Analysis

Linkage disequilibrium is the non-random association of alleles at two or more loci and represents a powerful tool for mapping genes involved in disease traits (see Ajioka R.S. et al., 1997). Biallelic markers, because they are densely spaced in the human genome and can be genotyped in greater numbers than other types of genetic markers (such as RFLP or VNTR markers), are particularly useful in genetic analysis based on linkage disequilibrium.

When a disease mutation is first introduced into a population (by a new mutation or the immigration of a mutation carrier), it necessarily resides on a single chromosome and thus on a single "background" or "ancestral" haplotype of linked markers. Consequently, there is complete disequilibrium between these markers and the disease mutation: one finds the disease mutation only in the presence of a specific set of marker alleles. Through subsequent generations recombination events occur between the disease mutation and these marker polymorphisms, and the disequilibrium gradually dissipates. The pace of this dissipation is a function of the recombination frequency, so the markers closest to the disease gene will manifest higher levels of disequilibrium than those that are further away. When not broken up

by recombination, "ancestral" haplotypes and linkage disequilibrium between marker alleles at different loci can be tracked not only through pedigrees but also through populations. Linkage disequilibrium is usually seen as an association between one specific allele at one locus and another specific allele at a second locus.

The pattern or curve of disequilibrium between disease and marker loci is expected to exhibit a maximum that occurs at the disease locus. Consequently, the amount of linkage disequilibrium between a disease allele and closely linked genetic markers may yield valuable information regarding the location of the disease gene. For fine-scale mapping of a disease locus, it is useful to have some knowledge of the patterns of linkage disequilibrium that exist between markers in the studied region. As mentioned above the mapping resolution achieved through the analysis of linkage disequilibrium is much higher than that of linkage studies. The high density of biallelic markers combined with linkage disequilibrium analysis provides powerful tools for fine-scale mapping. Different methods to calculate linkage disequilibrium are described below under the heading "Statistical Methods".

Population-Based Case-Control Studies Of Trait-Marker Associations

As mentioned above, the occurrence of pairs of specific alleles at different loci on the same chromosome is not random and the deviation from random is called linkage disequilibrium. Association studies focus on population frequencies and rely on the phenomenon of linkage disequilibrium. If a specific allele in a given gene is directly involved in causing a particular trait, its frequency will be statistically increased in an affected (trait positive) population, when compared to the frequency in a trait negative population or in a random control population. As a consequence of the existence of linkage disequilibrium, the frequency of all other alleles present in the haplotype carrying the trait-causing allele will also be increased in trait positive individuals compared to trait negative individuals or random controls. Therefore, association between the trait and any allele (specifically a biallelic marker allele) in linkage disequilibrium with the trait-causing allele will suffice to suggest the presence of a trait-related gene in that particular region. Case-control populations can be genotyped for biallelic markers to identify associations that narrowly locate a trait causing allele. As any marker in linkage disequilibrium with one given marker associated with a trait will be associated with the trait. Linkage disequilibrium allows the relative frequencies in case-control populations of a limited number of genetic polymorphisms (specifically biallelic markers) to be analyzed as an alternative to screening all possible functional polymorphisms in order to find trait-causing alleles. Association studies compare the frequency of marker alleles in unrelated case-control populations, and represent powerful tools for the dissection of complex traits.

Case-Control Populations (Inclusion Criteria)

Population-based association studies do not concern familial inheritance but compare the prevalence of a particular genetic marker, or a set of markers, in case-control populations. They are case-control studies based on comparison of unrelated case (affected or trait positive) individuals and unrelated control (unaffected, trait negative or random) individuals. Preferably the control group is composed of unaffected or trait negative individuals. Further, the control group is ethnically matched to the case population. Moreover, the control group is preferably matched to the case-population for the main known confusion factor for the trait under study (for example age-matched for an age-dependent trait). Ideally, individuals in the two samples are paired in such a way that they are expected to differ only in their disease status. The terms "trait positive population", "case population" and "affected population" are used interchangeably herein.

An important step in the dissection of complex traits using association studies is the choice of case-control populations (see Lander and Schork, 1994). A major step in the choice of case-control populations is the clinical definition of a given trait or phenotype. Any genetic trait may be analyzed by the association method proposed here by carefully selecting the individuals to be included in the trait positive and trait negative phenotypic groups. Four criteria are often useful: clinical phenotype, age at onset, family history and severity. The selection procedure for continuous or quantitative traits (such as blood pressure for example) involves selecting individuals at opposite ends of the phenotype distribution of the trait under study, so as to include in these trait positive and trait negative populations individuals with non-overlapping phenotypes. Preferably, case-control populations consist of phenotypically homogeneous populations. Trait positive and trait negative populations consist of phenotypically uniform populations of individuals representing each between 1 and 98%, preferably between 1 and 80%, more preferably between 1 and 50%, and more preferably between 1 and 30%, most preferably between 1 and 20% of the total population under study, and preferably selected among individuals exhibiting non-overlapping phenotypes. The clearer the difference between the two trait phenotypes, the greater the probability of detecting an association with biallelic markers. The selection of those drastically different but relatively uniform phenotypes enables efficient comparisons in association studies and the possible detection of marked differences at the genetic level, provided that the sample sizes of the populations under study are significant enough.

In preferred embodiments, a first group of between 50 and 300 trait positive individuals, preferably about 100 individuals, are recruited according to their phenotypes. A similar number of control individuals are included in such studies.

In the present invention, typical examples of inclusion criteria include, but are not restricted to, prostate cancer or aggressiveness of prostate cancer tumors. In one preferred embodiment of the present invention, association studies are carried out on the basis of a presence (trait positive) or absence (trait negative) of prostate cancer.

Associations studies can be carried out by the skilled technician using the biallelic markers of the invention defined above, with different trait positive and trait negative populations. Suitable further examples of association studies using biallelic markers of the *PCTA-1* gene, including the biallelic markers A1 to A125, involve studies on the following populations:

- a trait positive population suffering from a cancer and a healthy unaffected population, or
- a trait positive population suffering from prostate cancer treated with agents acting against prostate cancer and suffering from side-effects resulting from this treatment and an trait negative population suffering from prostate cancer treated with same agents without any substantial side-effects, or
- a trait positive population suffering from prostate cancer treated with agents acting against prostate cancer showing a beneficial response and a trait negative population suffering from prostate cancer treated with same agents without any beneficial response, or
- a trait positive population suffering from prostate cancer presenting highly aggressive prostate cancer tumors and a trait negative population suffering from prostate cancer with prostate cancer tumors devoid of aggressiveness.

Association Analysis

The general strategy to perform association studies using biallelic markers derived from a region carrying a candidate gene is to scan two groups of individuals (case-control populations) in order to measure and statistically compare the allele frequencies of the biallelic markers of the present invention in both groups.

If a statistically significant association with a trait is identified for at least one or more of the analyzed biallelic markers, one can assume that: either the associated allele is directly responsible for causing the trait (i.e. the associated allele is the trait causing allele), or more likely the associated allele is in linkage disequilibrium with the trait causing allele. The specific characteristics of the associated allele with respect to the candidate gene function usually give further insight into the relationship between the associated allele and the trait (causal or in linkage disequilibrium). If the evidence indicates that the associated allele within the candidate

gene is most probably not the trait causing allele but is in linkage disequilibrium with the real trait causing allele, then the trait causing allele can be found by sequencing the vicinity of the associated marker, and performing further association studies with the polymorphisms that are revealed in an iterative manner.

Association studies are usually run in two successive steps. In a first phase, the frequencies of a reduced number of biallelic markers from the candidate gene are determined in the trait positive and control populations. In a second phase of the analysis, the position of the genetic loci responsible for the given trait is further refined using a higher density of markers from the relevant region. However, if the candidate gene under study is relatively small in length, as is the case for *PCTA-1*, a single phase may be sufficient to establish significant associations.

It is another object of the present invention to provide a method for the identification and characterization of an association between an allele of one or more biallelic markers of a *PCTA-1* gene and a trait. The method comprises the steps of:

- genotyping a marker or a group of biallelic markers according to the invention in trait positive and control individuals; and

- establishing a statistically significant association between one allele of at least one marker and the trait.

The control individuals can be random or trait negative populations. Preferably, the trait positive and trait negative individuals are selected from non-overlapping phenotypes relating trait under study. In some embodiments, the biallelic marker is comprised in one or more of the sequences of P1 to P125, and the complementary sequences thereof.

The invention also comprises methods of detecting an association between a genotype and a phenotype, comprising the steps of a) determining the frequency of at least one *PCTA-1*-related biallelic marker in a trait positive population according to a genotyping method of the invention; b) determining the frequency of said *PCTA-1*-related biallelic marker in a control population according to a genotyping method of the invention; and c) determining whether a statistically significant association exists between said genotype and said phenotype. In addition, the methods of detecting an association between a genotype and a phenotype of the invention encompass methods with any further limitation described in this disclosure, or those following, specified alone or in any combination: Optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to

A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, said control population may be a trait negative population, or a random population; Optionally, each of said genotyping steps a) and b) may be performed on a pooled biological sample derived from each of said populations; Optionally, each of said genotyping of steps a) and b) is performed separately on biological samples derived from each individual in said population or a subsample thereof; Optionally, the identity of the nucleotides at the biallelic markers of the *PCTA-1* gene is determined in steps a) and b). Optionally, said phenotype is symptoms of, or susceptibility to cancer, preferably prostate cancer, the level of aggressiveness of prostate cancer tumors, an early onset of prostate cancer, a beneficial response to or side effects related to treatment against prostate cancer.

If the trait is a beneficial response or inversely a side effect to a treatment of prostate cancer, the method of the invention referred to above further comprises some or all of the following steps:

- selecting a population or cohort of subjects diagnosed as suffering from prostate cancer;

- administering a specified treatment of prostate cancer to said cohort of subjects;

- monitoring the outcome of drug administration and identifying those individuals that are trait positive or trait negative relative to the treatment;

- taking from said cohort biological samples containing DNA and testing this DNA for the presence of a specific allele or of a set of alleles of biallelic markers of the *PCTA-1* gene;

- analyzing the distribution of alleles of biallelic markers between trait positive and trait negative individuals; and

- performing a statistical analysis to determine a statistically significant association between the presence or absence of alleles of biallelic markers of the *PCTA-1* gene and the treatment related trait.

Haplotype Analysis

As described above, when a chromosome carrying a disease allele first appears in a population as a result of either mutation or migration, the mutant allele necessarily resides on a chromosome having a set of linked markers: the ancestral haplotype. This haplotype can be tracked through populations and its statistical association with a given trait can be analyzed. Complementing single point (allelic) association studies with multi-point association studies also called haplotype studies increases the statistical power of association studies. Thus, a haplotype association study allows one to define the frequency and the type of the ancestral carrier haplotype. A haplotype analysis is important in that it increases the statistical power of an analysis involving individual markers.

In a first stage of a haplotype frequency analysis, the frequency of the possible haplotypes based on various combinations of the identified biallelic markers of the invention is determined. The haplotype frequency is then compared for distinct populations of trait positive and control individuals. The number of trait positive individuals, which should be, subjected to this analysis to obtain statistically significant results usually ranges between 30 and 300, with a preferred number of individuals ranging between 50 and 150. The same considerations apply to the number of unaffected individuals (or random control) used in the study. The results of this first analysis provide haplotype frequencies in case-control populations, for each evaluated haplotype frequency a p-value and an odd ratio are calculated. If a statistically significant association is found the relative risk for an individual carrying the given haplotype of being affected with the trait under study can be approximated.

The present invention also provides a method for the identification and characterization of an association between a haplotype comprising alleles of several biallelic markers of the genomic sequence of the *PCTA-1* gene and a trait. The method comprises the steps of:

- genotyping a group of biallelic markers according to the invention in trait positive and control individuals; and
- establishing a statistically significant association between a haplotype and the trait.

Preferably, the control individuals can be random or trait negative populations. In some embodiments, the haplotype comprises two or more biallelic markers comprised in the sequences of P1 to P125, and the complementary sequences thereof.

An additional embodiment of the present invention encompasses methods of detecting an association between a haplotype and a phenotype, comprising the steps of: a) estimating the frequency of at least one haplotype in a trait positive population, according to a method of the invention for estimating the frequency of a haplotype; b) estimating the frequency of said haplotype in a control population, according to a method of the invention for estimating the

frequency of a haplotype; and c) determining whether a statistically significant association exists between said haplotype and said phenotype. In addition, the methods of detecting an association between a haplotype and a phenotype of the invention encompass methods with any further limitation described in this disclosure, or those following: Optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A1 to A44, A46 to A53, A57, A58, A62 to A76, A81, A82, A86 to A91, A107, A118, and A123 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A45, A54, A60, A61, A77 to A80, A83 to A85, A93, A102 to A106, A109, A110, A114, and A122, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; optionally, wherein said *PCTA-1*-related biallelic marker is selected from the group consisting of A55, A56, A59, A92, A94 to A101, A108, A111 to A113, A115 to A117, and A119 to A121, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith; Optionally, said control population is a trait negative population, or a random population. Optionally, said phenotype is symptoms of, or susceptibility to cancer, preferably prostate cancer, the level of aggressiveness of prostate cancer tumors, an early onset of prostate cancer, a beneficial response to or side effects related to treatment against prostate cancer; Optionally, said method comprises the additional steps of determining the phenotype in said trait positive and said control populations prior to step c).

Interaction Analysis

The biallelic markers of the present invention may also be used to identify patterns of biallelic markers associated with detectable traits resulting from polygenic interactions. The analysis of genetic interaction between alleles at unlinked loci requires individual genotyping using the techniques described herein. The analysis of allelic interaction among a selected set of biallelic markers with appropriate level of statistical significance can be considered as a haplotype analysis. Interaction analysis consists of stratifying the case-control populations with respect to a given haplotype for the first loci and performing a haplotype analysis with the second loci with each subpopulation.

Statistical methods used in association studies are further described below.

Testing For Linkage In The Presence Of Association

The biallelic markers of the present invention may further be used in TDT (transmission/disequilibrium test). TDT tests for both linkage and association and is not

affected by population stratification. TDT requires data for affected individuals and their parents or data from unaffected sibs instead of from parents (see Spielmann S. et al., 1993; Schaid D.J. et al., 1996, Spielmann S. and Ewens W.J., 1998). Such combined tests generally reduce the false – positive errors produced by separate analyses.

Association OF Biallelic Markers Of The Invention With Prostate Cancer

Trait Positive And Control Populations

Two groups of independent individuals were used: the overall trait positive and the trait negative populations included 491 individuals suffering from prostate cancer and 313 individuals without any sign of prostate cancer. A specific protocol for the collection of DNA samples from trait positive and trait negative individuals is described in Example 5. The 491 individuals suffering from prostate cancer can be subdivided into a population of individuals who developed prostate cancer under 65 years-old and a population of individuals who developed prostate cancer after the age of 65. The population of individuals who are less than 65 years-old was used to determine an association with an early onset of prostate cancer. The affected individuals can also be subdivided in familial cases and sporadic cases.

In order to have as much certainty as possible on the absence of prostate cancer in trait negative individuals, it is preferred to conduct a PSA dosage analysis on this population. Several commercial assays can be used (WO 96/21042, the disclosure of which is incorporated herein by reference in its entirety). In one preferred embodiment, a Hybritech assay is used and trait negative individuals must have a level of PSA less than 2.8 ng/ml of serum in order to be selected as such. In a preferred embodiment, the Yang assay is used and trait negative individuals must have a level of PSA of less than 4 ng/ml of serum in order to be included in the population under study.

Association Analysis

In one preferred embodiment of the invention in which a correlation was found between biallelic markers of the *PCTA-1* gene and prostate cancer, results of the association study, further details of which are provided in example 5, seem to indicate that prostate cancer, preferably familial prostate cancer, more preferably early onset familial prostate cancer, is associated most strongly with the biallelic markers A30 (99-1572/440) and A41 (5-171/204) which present a particular interest. These association results constitute new elements for studying the genetic susceptibility of individuals to prostate cancer, preferably to familial prostate cancer, more preferably familial early onset prostate cancer. Further details concerning this association study are provided below.

The biallelic markers most strongly associated with prostate cancer, namely A30 and A41, are located in the regulatory region of the *PCTA-1* gene, more particularly in the promoter region. The consequences of the presence of these markers in these regions are discussed below.

Furthermore, the biallelic marker A2 (99-1601/402) was found to be also associated with prostate cancer, more particularly with sporadic prostate cancer. This biallelic marker is localized in the 5' regulatory region of the *PCTA-1* gene.

Similar association studies can also be carried out with other biallelic markers within the scope of the invention, preferably with biallelic markers in linkage disequilibrium with the markers associated with prostate cancer as described above, including the biallelic markers A1 to A125.

Analysis Of Biallelic Marker Associations

Even though polymorphisms associated with prostate cancer have been identified in the coding region of the *PCTA-1* gene, these polymorphisms do not appear to be as significant as those found in the upstream regulatory region of the *PCTA-1* gene. The results further suggest that a trait-causing mutation is likely to be located within the 5' regulatory region of the *PCTA-1* gene. The extent to which the markers found within the coding region of *PCTA-1* are significant in relation to cancer can be determined using haplotype analyses involving at least two of the biallelic markers of the present invention.

Six of the biallelic markers of the present invention result in a change in the amino acid sequence of a PCTA-1 protein. These are biallelic markers A54, A56, A60, A75, A76 and A85. These mutations may change the function and/or the stability of the PCTA-1 protein. An amino acid change in a PCTA-1 protein can lead to alterations in PCTA-1 biological activity. Either a modified function or an increased stability can be involved in prostate cancer appearance.

Furthermore, as the expression of the *PCTA-1* gene has mainly been reported in prostate cancer cells, one can assume that its expression is closely linked to the development of cancer, particularly prostate cancer. Generally, a major control of gene expression proceeds at the level of the initiation of the transcription. This initiation involves the promoter which can be considered as a concentration of transcription factor binding sites. The initiation of the transcription also involves enhancers which modulate the efficiency of the initiation and consist of DNA binding sites which are located in regulatory regions of the considered gene which may be at a certain distance in 3' or 5' of the gene.

Most of the biallelic polymorphisms of the *PCTA-1* gene associated with prostate cancer according to the present invention are located in the regulatory region upstream of the

transcription start site of the *PCTA-1* gene and particularly in the promoter. Biallelic marker A41, which is located about 120 bp upstream of the beginning of the first exon (exon 0), may be comprised in the proximal promoter of the *PCTA-1* gene. This biallelic marker could be a trait causing mutation of prostate cancer. Biallelic marker A30, which is located about 1.5 kb upstream the beginning of the first exon (exon 0), may be comprised in the distal promoter of the *PCTA-1* gene. Biallelic marker A2 is located in the 5' regulatory region of the *PCTA-1* gene.

As the expression of the *PCTA-1* gene has mainly been reported in prostate cancer cells, the expression of *PCTA-1* gene is modified during the carcinogenesis. The exact mechanism through which PCTA expression is modified is not understood. However, it is possible that the polymorphisms A41, A30, and A2 modulate *PCTA-1* expression by modulating *PCTA-1* transcription through DNA binding proteins, which will be explained in further detail below.

The regulation of *PCTA-1* expression is a key factor in the onset and for development of cancer and particularly prostate cancer. In this regard, the polymorphisms located in the 5' regulatory region of the *PCTA-1* gene appear to play the most significant role in the association of *PCTA-1* with cancer. It appears clear that the polymorphisms found in the promoter region adjacent to the transcription initiation site, and particularly those located in the proximal *PCTA-1* promoter, are more strongly associated with prostate cancer than polymorphisms of the other promoter elements located further upstream of this site. Furthermore, some polymorphisms, such as the biallelic marker A41, are clearly associated with early onset prostate cancer. The polymorphisms found in the proximal 2000 to 3000 bp of the 5' regulatory region are associated with early onset prostate cancer. The inventors have also shown an association between some of the biallelic markers of the present invention located at the 3' end of the *PCTA-1* genomic DNA and prostate cancer.

The involvement of the associated polymorphisms in the modification of the *PCTA-1* expression in prostate cancer cells can be confirmed through the assays described below.

The expression levels of a *PCTA-1* gene, preferably a gene comprising at least one biallelic marker according to the invention, in different tissues, can be determined by analyses of tissue samples from individuals typed for the presence or absence of a specific polymorphism. Any convenient method can be used such as Northern, or Dot blot or other hybridization analyses, and quantitative RT-PCR for mRNA quantitation, Western blot ELISA, RIA for protein quantitation. The tissue specific expression can then be correlated with the genotype. More details on some of these methods are provided below under the heading "Method For Screening".

The effects of modifications in the regulatory regions of the *PCTA-1* gene, and particularly in the sequence of its promoter, can be studied through the determination of expression levels by expression assays for the particular promoter sequence. The assays are performed with the *PCTA-1* coding sequence or with a detectable marker sequence using a reporter gene. To determine tissue specificity, the assay is performed in cells from different sources. Preferably the assay is performed on normal tissue cells and cancerous cells of the same tissue type (e.g. prostate cells and on prostate cancer cells). More preferably, the assay is performed on a large range of cell lines with an increasing level of malignancy. Some methods are discussed in more detail below under the heading "Method For Screening".

An assay to determine the effect of a sequence polymorphism on *PCTA-1* expression may be performed in cell-free extracts, or in cell-culture assays, such as transient or stable transfection assays. This assay is also within the scope of the present invention. Alterations in expression may be correlated to decreases or increases in the basic amounts of *PCTA-1* mRNA and/or protein that are expressed in one or more cell types. Expression levels of different alleles are compared using various methods known in the art. Methods for determining whether the level of expression triggered by promoter or enhancer sequences is increased or decreased depending on the studied allele of said sequence include the insertion into a vector of said sequence upstream a reporter gene such as β-galactosidase, luciferase, green fluorescent protein or chloramphenicol acetyltransferase. Expression levels are assessed by quantitation of expressed reporter proteins that provides for convenient quantitation.

The changes in *PCTA-1* expression can be the result of modifications in the modulation of *PCTA-1* transcription by DNA binding proteins, which are able to activate or inhibit the initiation of the transcription of the *PCTA-1* gene. The term "DNA binding protein" is intended to encompass more particularly transcriptional factors. The binding of these proteins on the sites located in the promoter is critical for a correct binding of polymerases and consequently for the initiation of transcription. The binding of these proteins on the sites located in the 5' upstream regulatory regions modulates transcription.

The binding sites of DNA binding proteins, preferably transcription factors, are generally 6-20 nucleotides in length. A polymorphic site located in a transcription factor binding site may result in a difference of binding affinity of the said transcription factor between the two allele of the polymorphism. This difference of affinity could explain the changes of expression of the *PCTA-1* gene.

When one or more alleles of the biallelic markers of the *PCTA-1* gene associated with cancer are present in the genome of an individual since conception, there would be an event which provokes a drastic increase in the expression of *PCTA-1*. There are at least two possible

hypotheses that can be formulated to explain this event. Firstly, as cancer is the result of a succession of mutations, one mutation could lead to either the expression of a new DNA binding activity, or the overexpression of a DNA binding factor which binds to the site containing the polymorphism and which is involved in the transcription of the *PCTA-1* gene. Secondly the DNA binding factor readily binds to the site containing the polymorphism in normal cells where it is either unable to activate the transcription of *PCTA-1* or repressor of the *PCTA-1* transcription initiation. A mutation in the transcription factor can make the transcription factor either functional in the case of an activator or unfunctional in the case of a repressor. Likewise, a mutation in an additional protein can induce the binding of this protein which is needed by the DNA binding factor for activating the transcription of the *PCTA-1* gene.

In order to confirm the capacity of transcription factors to bind sites containing the biallelic markers of the present invention, so as to assess the difference in affinity between the two alleles of the considered biallelic marker and to discriminate between these hypotheses, a gel retardation assay or DNA mobility shift assay can be carried out. This type of assay is well-known to those skilled in the art and is described in US 5,698,389, US 5,502,176, Fried and Crothers (1981), Garner and Revzin (1981) and Dent and Latchman (1993).

This type of method relies on the principle that a fragment of DNA to which a protein has bound will move more slowly in gel electrophoresis than the same DNA fragment without the bound protein. The DNA mobility shift assay is carried out, therefore, by first labeling the specific DNA segment whose protein-binding properties are being investigated. The labeled DNA is then incubated with a nuclear (Dignam *et al.*, 1983; Schreiber et al., 1989; Muller et al., 1989; Mizokami et al., 1994) or whole cell (Manley *et al.*, 1980) extract of cells prepared in such a way as to contain DNA-binding proteins. DNA-protein complexes are then allowed to form. The complexes are then electrophoresed on a non-denaturing polyacrylamide gel and the position of the labeled DNA is visualized by suitable techniques. Various types of suitable labels can be selected by the person skilled in the art. Notably, the radioactive labeling is appropriate. If no protein has bound to the DNA, all the label is free to migrate quickly, whereas labeled protein-DNA complexes migrate more slowly and hence give a different signal from that of the unbound DNA near the top of the gel. The interaction specificity can be estimated by carrying out a gel retardation assay with increasing amount of unlabeled DNA segment which can compete with the labeled one. A positive control can be realized with an oligonucleotide containing the androgene responsive element.

The investigated DNA segment preferably comprises the sequence of a potential binding site containing an allele of a polymorphism of the present invention, more preferably a sequence comprising a sequence selected from P1 to P125 and the complementary sequences

thereto, still more preferably a sequence comprising a sequence selected from P1 to P43 and the complementary sequences thereto. In an embodiment, the polymorphism site is located in the middle of the DNA fragment. In an other embodiment, the polymorphism site can be located close to an end of the DNA fragment, for example at 6 nucleotides away from the end. The DNA fragment has a sufficient length to hybridize with the complementary strand and to form a stable double strand. For example, the DNA fragment comprises at least 8 nucleotides, preferably at least 20 nucleotides, more preferable 30 nucleotides. In a specific embodiment, the DNA fragment comprises the sequence of interest at the middle of the fragment and some poly G, poly C, or poly GC at its 5' and/or 3' ends.

In a preferred embodiment, the DNA segment consists of an oligonucleotide selected from the group consisting of Oligo1 to Oligo60 which are described in Table C and detailed as feature in SEQ ID No 1. For each polymorphic site, 4 oligonucleotides are generated and correspond to the two complementary strands of the DNA for each of the two alleles of the considered polymorphism. The DNA segments are designed such as the polymorphic base is surrounded with 14 nucleotides on each side.

Table C

Biallelic marker	All	Oligonu-cleotide name	Position range of the oligonucleotide in SEQ ID No 1		Oligonu-cleotide name	Complementary position range of the oligonucleotide in SEQ ID No 1	
			Beginning	End		Beginning	End
5-169-208	A	Oligo1	67820	67848	Oligo31	67820	67850
5-169-208	G	Oligo2	67820	67848	Oligo32	67820	67850
5-169-331	C	Oligo3	67940	67969	Oligo33	67941	67969
5-169-331	T	Oligo4	67940	67969	Oligo34	67941	67969
5-169-97	C	Oligo5	67707	67737	Oligo35	67709	67738
5-169-97	G	Oligo6	67707	67737	Oligo36	67709	67738
5-170-238	A	Oligo7	68198	68227	Oligo37	68199	68228
5-170-238	G	Oligo8	68198	68227	Oligo38	68199	68228
5-170-288	A	Oligo9	68247	68277	Oligo39	68249	68277
5-170-288	C	Oligo10	68247	68277	Oligo40	68249	68277
5-171-156	G	Oligo11	68463	68491	Oligo41	68463	68492
5-171-156	T	Oligo12	68463	68491	Oligo42	68463	68492
5-171-204	C	Oligo13	68511	68539	Oligo43	68511	68539
5-171-204	T	Oligo14	68511	68539	Oligo44	68511	68539
5-171-273	A	Oligo15	68580	68608	Oligo45	68580	68608
5-171-273	G	Oligo16	68580	68608	Oligo46	68580	68608
5-171-289	C	Oligo17	68596	68624	Oligo47	68596	68626
5-171-289	T	Oligo18	68596	68624	Oligo48	68596	68626
5-171-54	C	Oligo19	68360	68389	Oligo49	68361	68389
5-171-54	G	Oligo20	68360	68389	Oligo50	68361	68389
99-1572-315	C	Oligo21	66951	66981	Oligo51	66953	66983
99-1572-315	T	Oligo22	66951	66981	Oligo52	66953	66983
99-1572-335	A	Oligo23	66973	67001	Oligo53	66973	67002

99-1572-335	G	Oligo24	66973	67001	Oligo54	66973	67002
99-1572-440	C	Oligo25	67078	67106	Oligo55	67078	67106
99-1572-440	T	Oligo26	67078	67106	Oligo56	67078	67106
99-1572-477	A	Oligo27	67113	67143	Oligo57	67115	67144
99-1572-477	T	Oligo28	67113	67143	Oligo58	67115	67144
99-1572-578	C	Oligo29	67212	67243	Oligo59	67215	67247
99-1572-578	T	Oligo30	67212	67243	Oligo60	67215	67247

Each oligonucleotide selected from Oligo1 to Oligo60 comprises 4 additional bases, namely GATC, at its 5' end.

In a preferred embodiment, either the nuclear or whole cell extracts are provided from normal and cancer cells, particularly from normal prostate cells and prostate cancer cells. For example, suitable cell extracts can be provided from PZ-HPV-7 (ATCC : CRL-2221), CA-HPV-10 (ATCC : CRL-2220), PC-3 (ATCC : CRL-1435), DU 145 (ATCC : HTB-81), LNCaP-FGC (ATCC : CRL-10995 and CRL-1740), or NCI-H660 (ATCC : CRL-5813) cells. In a more preferred embodiment, the cell extracts are provided form PNT1A, PNT2, LNCaP-JMV, DU145 (ATCC Nr : HTB-81) or PC3 (ATCC Nr : CRL-1435) cells.

In case a new transcription factor is specifically expressed in cancer cells, a gel retardation assay will show a retarded or shifted band only when the DNA was incubated with cell extracts from prostate cancer cells. If the DNA binding activity already exists in normal cells, the gel retardation assay will show a shifted band with cell extracts from normal prostate cells and prostate cancer cells. Gel retardation assays will also allow to show a significant difference in affinity between a DNA binding factor and binding sites containing the two alleles of the considered polymorphism.

The interaction of the DNA segment described above with transcription factors can also be studied with an optical biosensor such as BIACORE. This technology is well-known to those skilled in the art and is described in Szabo et al. (1995) and Edwards et al. (1997). The main advantage of this method is that it allows the determination of the association rate between the DNA fragment which is investigated and the DNA binding protein. Typically, a DNA segment such as those defined above is biotinylated at its 5' or 3' ends and is immobilized on a streptavidin-coated sensor chip. Then, a whole or a nuclear extract of cells is placed in contact with the DNA segment. The binding of DNA binding proteins to the DNA fragment causes a change in the refractive index and/or thickness. This change is detected by the Biosensor provided it occurs in the evanescent field. The affinity of the DNA binding protein to the DNA fragment can then be measured.

In order to precisely localize the binding site of the transcription factors, DNAse I footprinting or DMS protection footprinting assays can also be carried out with DNA fragments

which contain the sequence of a potential binding site containing an allele of a polymorphism of the present invention, preferably a sequence comprising a sequence selected from P1 to P125 and the complementary sequences thereto, more preferably a sequence comprising a sequence selected from P1 to P43 and the complementary sequences thereto. This type of assay is well-known to those skilled in the art and is described in Galas and Schmitz (1978), and Dynan and Tjian (1983). Briefly, in the DNAse I footprinting assay, end-labeled DNA is incubated with protein extract and then partially digested with DNAse I. Specific binding of proteins to DNA will modify nuclease digestion at the site of interaction relative to free DNA, leaving an "imprint" which can be visualized after extraction of the labeled DNA and electrophoresis in a sequence gel.

The interaction with transcription factors can also be studied with the methylation interference assay which is well-known to those skilled in the art and is described in Siebenlist and Gilbert (1980) and Maxam and Gilbert (1980). Briefly, this method relies on the ability of DMS to methylate G residues, which can be cleaved with piperidine. The target DNA is partially methylated so that, on average, only one G residue per DNA molecule is methylated. These partially methylated molecules is used in a DNA mobility shift experiment with an appropriate cell extract containing transcription factors. After electrophoresis, the band produced by the DNA which has bound protein and that produced by the unbound DNA are excised from the gel and treated with piperidine to cleave the DNA at the methylated G residues and not at unmethylated G residues. If methylation of a particular G residue prevents transcription factors binding, then cleavage at this methylated G residue will be observed only in the DNA that failed to bind the protein.

In order to confirm the implication of a particular *PCTA-1* derived sequence containing the biallelic marker as a binding site for a transcription regulator of *PCTA-1* in cancer cells, a transient expression assay can be carried out in which a vector comprising the considered binding site upstream of the HSV1 thymidine kinase promoter operably linked to a reporter gene such as chloramphenicol acetyltransferase is transfected in appropriate cell lines. This assay is well-known to those skilled in the art and is described in Doucas et al. (1991). This assay can also be realized by cloning the considered binding site upstream the SV40 promoter into the pGL3-promoter luciferase vector (Promega) as described in Coles et al. (1998). Both normal and cancer cells, more particularly normal and cancer cells from prostate, are transfected with said vector. The effect of the binding site and more particularly of the alleles comprised in the binding site can be assessed through the expression level of the reporter gene.

The inventors believe that these polymorphisms, particularly the polymorphisms located on or close to polyadenylation sites have a direct although somewhat milder effect on prostate cancer development.

Haplotype Analysis

In the context of the present invention, a haplotype can be defined as a combination of biallelic markers found in a given individual and which may be associated more or less significantly, as a result of appropriate statistical analyses, with the expression of a given trait.

A two-marker haplotype including markers A30 and A41 (TT alleles respectively) was shown to be significantly associated with prostate cancer, preferably with a familial prostate cancer, more preferably with a familial early onset prostate cancer. As shown in Table 8, the "TT" haplotype present a p-value of 2.5×10^{-6} for the familial early onset prostate cancer (see Example 5).

A three-marker haplotype including markers A2, A30, and A41 (ATT alleles respectively) was shown to be significantly associated with prostate cancer, preferably with a familial prostate cancer, more preferably with a familial early onset prostate cancer. As shown in table 8, the "ATT" haplotype present a p-value of 2.5×10^{-7} for the familial early onset prostate cancer (see Example 5).

A first two-marker haplotype including markers A2 and A57 (99-1605/112) (TA alleles, respectively) was shown to be significantly associated with prostate cancer, preferably with a sporadic prostate cancer. As shown in table 8, the "TA" haplotype present a p-value of 3.4×10^{-5} for the sporadic informative prostate cancer (see Example 5). A second two-marker haplotype including markers A2 and A55 (5-2/178) (TT alleles, respectively) was shown to be significantly associated with prostate cancer, preferably with a sporadic prostate cancer. As shown in table 8, the "TT" haplotype present a p-value of 1×10^{-5} for the sporadic informative prostate cancer (see Example 5).

Therefore, one preferred haplotype of the present invention associated with a familial prostate cancer comprises a biallelic marker selected from the group consisting of A30 (allele T), A41 (allele T), A2 (allele A), A55 (allele C) and A57 (allele G). One more preferred haplotype of the present invention associated with a familial prostate cancer comprises a biallelic marker selected from the group consisting of A30 (allele T), A41 (allele T), and A2 (allele A). One still more haplotype of the present invention associated with a familial prostate cancer comprises a biallelic marker selected from the group consisting of A30 (allele T), and A41 (allele T).

Furthermore, one preferred haplotype of the present invention associated with a sporadic prostate cancer comprises a biallelic marker selected from the group consisting of A2 (allele T), A55 (allele T), A57 (allele A), A30 (allele T) and A41 (allele T). One more preferred haplotype of the present invention associated with a sporadic prostate cancer comprises a biallelic marker selected from the group consisting of A2 (allele T), A41 (allele T), A55 (allele T), A57 (allele A).

The permutation tests clearly validated the statistical significance of the association between these haplotypes and the prostate cancer (see Example 5). All these haplotypes can be used in diagnostic of prostate cancer, more particularly either familial prostate cancer or sporadic prostate cancer.

One can observe that the haplotypes associated to familial cases of prostate cancer are not associated with the sporadic cases of prostate cancer and that the haplotypes associated to the sporadic cases are not associated with the familial cases (see Table 7 of Example 5). Moreover, except the biallelic markers A2, the familial and sporadic cases haplotypes do not present any common biallelic marker. Therefore, the ancestral haplotypes would be different and the causing trait allele would not be the same.

This information is extremely valuable. The knowledge of a potential genetic predisposition to prostate cancer, even if this predisposition is not absolute, might contribute in a very significant manner to treatment efficacy of prostate cancer and to the development of new therapeutic and diagnostic tools.

Statistical methods

In general, any method known in the art to test whether a trait and a genotype show a statistically significant correlation may be used.

1) Methods In Linkage Analysis

Statistical methods and computer programs useful for linkage analysis are well-known to those skilled in the art (see Terwilliger J.D. and Ott J., 1994; Ott J., 1991).

2) Methods To Estimate Haplotype Frequencies In A Population

As described above, when genotypes are scored, it is often not possible to distinguish heterozygotes so that haplotype frequencies cannot be easily inferred. When the gametic phase is not known, haplotype frequencies can be estimated from the multilocus genotypic data. Any method known to person skilled in the art can be used to estimate haplotype frequencies (see Lange K., 1997; Weir, B.S., 1996) Preferably, maximum-likelihood haplotype frequencies are computed using an Expectation- Maximization (EM) algorithm (see Dempster et al., 1977;

Excoffier L. and Slatkin M., 1995). This procedure is an iterative process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multi-locus genotype data when the gametic phase is unknown. Haplotype estimations are usually performed by applying the EM algorithm using for example the EM-HAPLO program (Hawley M. E. et al., 1994) or the Arlequin program (Schneider et al., 1997). The EM algorithm is a generalized iterative maximum likelihood approach to estimation and is briefly described below.

Please note that in the present section, "Methods To Estimate Haplotype Frequencies In A Population, " of this text, phenotypes will refer to multi-locus genotypes with unknown phase. Genotypes will refer to known-phase multi-locus genotypes.

A sample of N unrelated individuals is typed for K markers. The data observed are the unknown-phase K-locus phenotypes that can categorized in F different phenotypes. Suppose that we have H underlying possible haplotypes (in case of K biallelic markers, $H=2^K$).

For phenotype j, suppose that c_j genotypes are possible. We thus have the following equation

$$P_j = \sum_{i=1}^{c_j} pr(genotype_i) = \sum_{i=1}^{c_j} pr(h_k, h_l) \qquad \text{Equation 1}$$

where Pj is the probability of the phenotype j, h_k and h_l are the two haplotypes constituent the genotype i. Under the Hardy-Weinberg equilibrium, $pr(h_k h_l)$ becomes:

$$pr(h_k, h_l) = pr(h_k)^2 \text{ if } h_k = h_l,\ pr(h_k, h_l) = 2pr(h_k).pr(h_l) \text{ if } h_k \neq h_l. \quad \text{Equation 2}$$

The successive steps of the E-M algorithm can be described as follows:

Starting with initial values of the of haplotypes frequencies, noted $p_1^{(0)}, p_2^{(0)}, \ldots . p_H^{(0)}$, these initial values serve to estimate the genotype frequencies (Expectation step) and then estimate another set of haplotype frequencies (Maximization step), noted $p_1^{(1)}, p_2^{(1)}, \ldots . p_H^{(1)}$, these two steps are iterated until changes in the sets of haplotypes frequency are very small.

A stop criterion can be that the maximum difference between haplotype frequencies between two iterations is less than 10^{-7}. These values can be adjusted according to the desired precision of estimations.

At a given iteration s, the Expectation step consists of calculating the genotypes frequencies by the following equation:

$$pr(genotype_i)^{(s)} = pr(phenotype_j).pr(genotype_i | phenotype_j)^{(s)}$$
$$= \frac{n_j}{N} \cdot \frac{pr(h_k, h_l)^{(s)}}{P_j^{(s)}} \qquad \text{Equation 3}$$

where genotype i occurs in phenotype j, and where h_k and h_l constitute genotype i. Each probability is derived according to eq. 1, and eq. 2 described above.

Then the Maximization step simply estimates another set of haplotype frequencies given the genotypes frequencies. This approach is also known as the gene-counting method (Smith, 1957).

$$p_t^{(s+1)} = \frac{1}{2} \sum_{j=1}^{F} \sum_{i=1}^{c_j} \delta_{it} . pr(genotype_i)^{(s)} \qquad \text{Equation 4}$$

Where δ_{it} is an indicator variable which count the number of time haplotype t in genotype i. It takes the values of 0, 1 or 2.

To ensure that the estimation finally obtained is the maximum-likelihood estimation several values of departures are required. The estimations obtained are compared and if they are different the estimations leading to the best likelihood are kept.

3) Methods To Calculate Linkage Disequilibrium Between Markers

A number of methods can be used to calculate linkage disequilibrium between any two genetic positions, in practice linkage disequilibrium is measured by applying a statistical association test to haplotype data taken from a population.

Linkage disequilibrium between any pair of biallelic markers comprising at least one of the biallelic markers of the present invention (M_i, M_j) having alleles (a_i/b_i) at marker M_i and alleles (a_j/b_j) at marker M_j can be calculated for every allele combination (a_i,a_j; a_i,b_j; b_i,a_j and b_i,b_j), according to the Piazza formula:

$\Delta_{aiaj} = \sqrt{\theta 4} - \sqrt{(\theta 4 + \theta 3)(\theta 4 + \theta 2)}$, where:

$\theta 4$= - - = frequency of genotypes not having allele a_i at M_i and not having allele a_j at M_j

$\theta 3$= - + = frequency of genotypes not having allele a_i at M_i and having allele a_j at M_j

$\theta 2$= + - = frequency of genotypes having allele a_i at M_i and not having allele a_j at M_j

Linkage disequilibrium (LD) between pairs of biallelic markers (M_i, M_j) can also be calculated for every allele combination (ai,aj; ai,bj; b_i,a_j and b_i,b_j), according to the maximum-likelihood estimate (MLE) for delta (the composite genotypic disequilibrium coefficient), as described by Weir (Weir B. S., 1996). The MLE for the composite linkage disequilibrium is:

$D_{aiaj} = (2n_1 + n_2 + n_3 + n_4/2)/N - 2(pr(a_i).\ pr(a_j))$

Where $n_1 = \Sigma$ phenotype (a_i/a_i, a_j/a_j), $n_2 = \Sigma$ phenotype (a_i/a_i, a_j/b_j), $n_3 = \Sigma$ phenotype (a_i/b_i, a_j/a_j), n4= Σ phenotype (a_i/b_i, a_j/b_j) and N is the number of individuals in the sample.

This formula allows linkage disequilibrium between alleles to be estimated when only genotype, and not haplotype, data are available.

Another means of calculating the linkage disequilibrium between markers is as follows. For a couple of biallelic markers, M_i (a_i/b_i) and M_j (a_j/b_j), fitting the Hardy-Weinberg equilibrium, one can estimate the four possible haplotype frequencies in a given population according to the approach described above.

The estimation of gametic disequilibrium between *ai* and *aj* is simply:

$$D_{aiaj} = pr(haplotype(a_i, a_j)) - pr(a_i).pr(a_j).$$

Where $pr(a_i)$ is the probability of allele a_i and pr(a_j) is the probability of allele a_j and where $pr(haplotype\ (a_i, a_j))$ is estimated as in Equation 3 above.

For a couple of biallelic marker only one measure of disequilibrium is necessary to describe the association between M_i and M_j.

Then a normalized value of the above is calculated as follows:

$$D'_{aiaj} = D_{aiaj} / \max(-pr(a_i).\ pr(a_j),\ -pr(b_i).\ pr(b_j)) \text{ with } D_{aiaj}<0$$

$$D'_{aiaj} = D_{aiaj} / \max(pr(b_i).\ pr(a_j),\ pr(a_i).\ pr(b_j)) \text{ with } D_{aiaj}>0$$

The skilled person will readily appreciate that other linkage disequilibrium calculation methods can be used.

Linkage disequilibrium among a set of biallelic markers having an adequate heterozygosity rate can be determined by genotyping between 50 and 1000 unrelated individuals, preferably between 75 and 200, more preferably around 100.

4) Testing For Association

Methods for determining the statistical significance of a correlation between a phenotype and a genotype, in this case an allele at a biallelic marker or a haplotype made up of such alleles, may be determined by any statistical test known in the art and with any accepted threshold of statistical significance being required. The application of particular methods and thresholds of significance are well with in the skill of the ordinary practitioner of the art.

Testing for association is performed by determining the frequency of a biallelic marker allele in case and control populations and comparing these frequencies with a statistical test to determine if their is a statistically significant difference in frequency which would indicate a correlation between the trait and the biallelic marker allele under study. Similarly, a haplotype analysis is performed by estimating the frequencies of all possible haplotypes for a given set of biallelic markers in case and control populations, and comparing these frequencies with a statistical test to determine if their is a statistically significant correlation between the haplotype

and the phenotype (trait) under study. Any statistical tool useful to test for a statistically significant association between a genotype and a phenotype may be used. Preferably the statistical test employed is a chi-square test with one degree of freedom. A P-value is calculated (the P-value is the probability that a statistic as large or larger than the observed one would occur by chance).

Statistical Significance

In preferred embodiments, significance for diagnosis purposes, either as a positive basis for further diagnostic tests or as a preliminary starting point for early preventive therapy, the p value related to a biallelic marker association is preferably about 1×10^{-2} or less, more preferably about 1×10^{-4} or less, for a single biallelic marker analysis and about 1×10^{-3} or less, still more preferably 1×10^{-6} or less and most preferably of about 1×10^{-8} or less, for a haplotype analysis involving two or more markers. These values are believed to be applicable to any association studies involving single or multiple marker combinations.

The skilled person can use the range of values set forth above as a starting point in order to carry out association studies with biallelic markers of the present invention. In doing so, significant associations between the biallelic markers of the present invention and prostate cancer, the level of aggressiveness of prostate cancer tumors, an early onset of prostate cancer, or a beneficial response to or side effects related to treatment against prostate cancer can be revealed and used for diagnosis and drug screening purposes.

Phenotypic Permutation

In order to confirm the statistical significance of the first stage haplotype analysis described above, it might be suitable to perform further analyses in which genotyping data from case-control individuals are pooled and randomized with respect to the trait phenotype. Each individual genotyping data is randomly allocated to two groups, which contain the same number of individuals as the case-control populations used to compile the data obtained in the first stage. A second stage haplotype analysis is preferably run on these artificial groups, preferably for the markers included in the haplotype of the first stage analysis showing the highest relative risk coefficient. This experiment is reiterated preferably at least between 100 and 10000 times. The repeated iterations allow the determination of the probability to obtain by chance the tested haplotype.

Assessment Of Statistical Association

To address the problem of false positives similar analysis may be performed with the same case-control populations in random genomic regions. Results in random regions and the

candidate region are compared as described in a co-pending US Provisional Patent Application entitled "Methods, Software And Apparati For Identifying Genomic Regions Harboring A Gene Associated With A Detectable Trait," U.S. Serial Number 60/107,986, filed November 10, 1998.

5) Evaluation Of Risk Factors

The association between a risk factor (in genetic epidemiology the risk factor is the presence or the absence of a certain allele or haplotype at marker loci) and a disease is measured by the odds ratio (OR) and by the relative risk (RR). If $P(R^+)$ is the probability of developing the disease for individuals with R and $P(R^-)$ is the probability for individuals without the risk factor, then the relative risk is simply the ratio of the two probabilities, that is:

$$RR= P(R^+)/P(R^-)$$

In case-control studies, direct measures of the relative risk cannot be obtained because of the sampling design. However, the odds ratio allows a good approximation of the relative risk for low-incidence diseases and can be calculated:

$$OR= (F^+/(1-F^+))/(F^-/(1-F^-))$$

F^+ is the frequency of the exposure to the risk factor in cases and F^- is the frequency of the exposure to the risk factor in controls. F^+ and F^- are calculated using the allelic or haplotype frequencies of the study and further depend on the underlying genetic model (dominant, recessive, additive...).

One can further estimate the attributable risk (AR) which describes the proportion of individuals in a population exhibiting a trait due to a given risk factor. This measure is important in quantifying the role of a specific factor in disease etiology and in terms of the public health impact of a risk factor. The public health relevance of this measure lies in estimating the proportion of cases of disease in the population that could be prevented if the exposure of interest were absent. AR is determined as follows:

$$AR = P_E (RR-1) / (P_E (RR-1)+1)$$

AR is the risk attributable to a biallelic marker allele or a biallelic marker haplotype. P_E is the frequency of exposure to an allele or a haplotype within the population at large; and RR is the relative risk which, is approximated with the odds ratio when the trait under study has a relatively low incidence in the general population.

Identification Of Biallelic Markers In Linkage Disequilibrium With The PCTA-1-Related Biallelic Markers

Once an association has been demonstrated between a given biallelic marker and a trait, the discovery of additional biallelic markers associated to trait and in linkage disequilibrium with one of the biallelic markers disclosed herein can easily be carried out by the skilled person.

The present invention then also concerns biallelic markers in linkage disequilibrium with the specific biallelic markers described above, more particularly with biallelic markers A1 to A125, and which are expected to present similar characteristics in terms of their respective association with a given trait.

Hence, once linkage disequilibrium has been demonstrated between a trait and a given biallelic marker, all the biallelic markers shown to be in linkage disequilibrium with the given biallelic marker are expected to present similar characteristics in terms of their respective association with a given trait. The discovery of additional biallelic markers associated with this trait is of great interest in order to increase the density of biallelic markers in this particular region because the causal mutation will be found in the vicinity of the marker or set of markers showing the highest correlation with the trait. These additional markers which can be identified and sequenced by the skilled person using the teachings of the present application also fall within the scope of the present invention.

The invention also concerns a method for the identification and characterization of a biallelic marker in linkage disequilibrium with a biallelic marker of the *PCTA-1* gene, preferably a biallelic marker of the *PCTA-1* gene of which one allele is associated with a trait. In one embodiment, the biallelic marker of the *PCTA-1* gene is outside of the *PCTA-1* gene itself. In another embodiment, the biallelic marker in linkage disequilibrium with a biallelic marker of the *PCTA-1* gene is itself located within the *PCTA-1* gene. The method comprises the following steps: (a) amplifying a genomic fragment, preferably comprising a first biallelic marker, from a plurality of individuals; (b) identifying second biallelic markers in said amplified portion; (c) conducting a linkage disequilibrium analysis between said first biallelic marker and second biallelic markers; and, (d) identifying second biallelic markers in linkage disequilibrium with said first marker. Subcombinations comprising steps (b) and (c) are also contemplated. Optionally, the first biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof. Preferably, the first biallelic marker is selected from the group consisting of A2, A30, A41, A55, A57 and the complements thereof.

Methods to identify biallelic markers and to conduct linkage disequilibrium analysis are described herein and can be carried out by the skilled person without undue experimentation.

Once identified, the sequences in linkage disequilibrium with a biallelic marker of the *PCTA-1* gene may be used in any of the methods described herein, including methods for determining an association between a biallelic marker and a trait, methods for identifying individuals having a predisposition for a trait, methods of administration of prophylactic or therapeutic agents disease treatment, methods of identifying individuals likely to respond positively or negatively to said agents, and methods of using drugs and vaccines.

An example of identification of additional biallelic markers associated to a trait based on the previous knowledge of the localization of a first marker associated to a given trait is given below.

Biallelic markers in linkage disequilibrium with a particular marker :Apo E4

The following example relating to the identification of markers in linkage disequilibrium with the apoE4 allele is representative of the procedures of the present invention in which markers in LD with a target gene are identified. 3 major isoforms of human apolipoprotein E (apoE2, -E3, and -E4) have been identified by isoelectric focusing and are coded for by 3 alleles (ε 2, 3, and 4) of the Apo E gene. As originally reported by Strittmatter et al. and by Saunders et al. in 1993, the Apo E ε4 allele is strongly associated with both late-onset familial and sporadic Alzheimer's Disease (AD).

Biallelic markers in linkage disequilibrium with the Apo E ε4 allele were identified. This example is illustrative of the general principle that the generation of biallelic markers associated with a trait leads to markers in linkage disequilibrium with any biallelic marker already known to be associated with the trait.

An Apo E marker was used to screen the human genomic BAC library. A BAC, which gave a unique hybridization signal on chromosomal region 19q13.2.3 by FISH, was selected for finding biallelic markers.

This BAC contained an insert of 205 kb that was subcloned. Fifty BAC subclones were randomly selected and sequenced. Twenty-five subclone sequences were selected and used to design twenty-five couples of PCR primers that allowed amplicons of approximately 500 bp to be generated. These PCR primers were then used to amplify the corresponding genomic sequences in a pool of DNA from 100 individuals (French origin, blood donors) as already described. Amplification products from pooled DNA were sequenced and analyzed for the presence of biallelic polymorphisms using the software described herein. Five amplicons were shown to contain a polymorphic base in the pool of 100 individuals, and therefore these polymorphisms (99-366/274; 99-344/439; 99-365/344; 99-359/308; 99-355/219) were selected as the random biallelic markers in the vicinity of the Apo E gene.

An additional couple of primers was designed that allowed amplification of the genomic fragment carrying the already known polymorphism of Apo E, (99-2452/54 C/T).

An association study was then performed. As expected, there was a clear association between Alzheimer disease (AD) and the known Apo E4 polymorphism (biallelic marker 99-2452/54), the C allele frequency being increased in 26 % in the AD case population studied compared to the AD control population analyzed (pvalue of this difference = $2x10^{-21}$).

In addition, the association study with the random markers generated in the vicinity of the Apo E gene showed that the biallelic marker 99-365/344 C/T is also associated to AD, the T allele frequency being increased of 17 % in the AD case population respect to the AD control population under study (pvalue of this allele frequency difference = $7x10^{-10}$). Thus individuals who possess a T allele at the biallelic marker 99-365/344 are at risk of developing AD.

Among the biallelic markers generated in the Apo E region, 99-365/344 is in LD with the previously known Apo E4 marker 99-2452/54. The linkage disequilibrium is detected in a control population (LD value = 0.08) and is clearly increased in the AD case population (LD = 0.21). Hence the generated biallelic marker which are associated with Alzheimer's disease, namely the biallelic marker 99-365, is in linkage disequilibrium with the biallelic marker 99-2452 already known to be associated with this disease.

Identification Of A Trait Causing Mutation In The *PCTA-1* Gene

If a statistically significant association with a trait is identified for at least one or more of the analyzed *PCTA-1*-related biallelic markers, one can assume that: either the associated allele is directly responsible for causing the trait, or more likely the associated allele is in linkage disequilibrium with the trait causing allele. More probably, the trait causing mutation would be found near to the associated biallelic markers.

Mutations in the *PCTA-1* gene which are responsible for a detectable phenotype may be identified by comparing the sequences of the *PCTA-1* gene from trait positive and trait negative individuals. Preferably, trait positive individuals to be sequenced carry a single marker allele or a haplotype shown to be associated to the trait and trait negative individuals to be sequenced do not carry such allele or haplotype associated to the trait. The detectable phenotype may comprise cancer, preferably prostate cancer, a response to or side effects related to a prophylactic or curative agent acting against prostate cancer, the aggressiveness of prostate cancer tumors, expression of the *PCTA-1* gene, a modified or forthcoming production of the PCTA-1 protein, or the production of a modified PCTA-1 protein. The mutations may comprise point mutations, deletions, or insertions in the *PCTA-1* gene. These mutations are called trait causing mutations and are at least partly responsible for a particular detectable phenotype in an

individual. The mutations may lie within the coding sequence for the PCTA-1 protein or within intronic and/or within regulatory regions in the *PCTA-1* gene, including splice sites, 5' UTRs, 3' UTRs and promoter sequences, including one or more transcription factor binding sites.

A further embodiment of the invention is a method to identify a trait causing mutation in the *PCTA-1* gene pursuant to the detection of an association between alleles of one or several of the biallelic markers of the present invention and a particular trait.

This method comprises the following steps :

- amplifying a region of the *PCTA-1* gene comprising a biallelic marker or a group of biallelic markers associated to the considered trait from DNA samples of trait positive and trait negative individuals;

- sequencing the amplified region;

- comparing DNA sequences from trait positive and trait negative individuals; and

- determining mutations specific to trait positive patients.

In some embodiments, the amplified region is a region located close to a biallelic marker of *PCTA-1* gene. In further embodiments, the amplified region is located close to one or more of the biallelic markers A1 to A125 and the complements thereof. In a preferred embodiment, the amplified region is located close to one or more of the biallelic markers A2, A30, A41, A55, A57 and the complements thereof.

Oligonucleotide primers are constructed as described previously to amplify the sequences of each of the exons, introns, the promoter region and the regulatory regions of the *PCTA-1* gene. Amplification is carried out on genomic DNA samples from trait positive patients and trait negative controls, preferably using the PCR conditions described in the examples. Amplification products from the genomic PCRs are then subjected to sequencing, preferably through automated dideoxy terminator sequencing reactions and electrophoresed, preferably on ABI 377 sequencers. Following gel image analysis and DNA sequence extraction, ABI sequence data are automatically analyzed to detect the presence of sequence variations among trait positive and trait negative individuals. Sequences are verified by determining the sequences of both DNA strands for each individual.

Candidate polymorphisms suspected of being responsible for the detectable phenotype, are then verified by screening a larger population of trait positive and trait negative individuals using polymorphism analysis techniques such as the techniques described above. Polymorphisms which exhibit a statistically significant correlation with the detectable phenotype are deemed responsible for the detectable phenotype.

The invention also concerns a mutated *PCTA-1* gene comprising a trait causing mutation, and particularly the mutated genes obtained by the process described above.

A mutated *PCTA-1* gene can be defined as a gene encoding either a modified or native PCTA-1 protein through a nucleotide sequence which is different from the nucleotide sequence of the *PCTA-1* gene found in a majority of trait negative individuals.

The region of the *PCTA-1* gene containing the mutation responsible for the detectable phenotype may be used in diagnostic techniques such as those described below. For example, microsequencing oligonucleotides, or oligonucleotides containing the mutation responsible for the detectable phenotype for amplification, or hybridization based diagnostics, such as those described herein, may be used for detecting individuals suffering from the detectable phenotype or individuals at risk of developing the detectable phenotype at a subsequent time. In addition, the *PCTA-1* allele responsible for the detectable phenotype may be used in gene therapy. The *PCTA-1* allele responsible for the detectable phenotype may also be cloned into an expression vector to express the mutant PCTA-1 protein as described herein.

Biallelic Markers Of The Invention In Methods Of Genetic Diagnostics

The biallelic markers of the present invention can also be used to develop diagnostics tests capable of identifying individuals who express a detectable trait as the result of a specific genotype or individuals whose genotype places them at risk of developing a detectable trait at a subsequent time. The trait analyzed using the present diagnostics may be any detectable trait, including susceptibility to cancer, preferably prostate cancer, the level of aggressiveness of prostate cancer tumors, an early onset of prostate cancer, a beneficial response to or side effects related to treatment against prostate cancer.

Information resulting from single marker association and for haplotype analyses is extremely valuable as it can, in certain circumstances, be used to initiate preventive treatments or to allow an individual carrying a significant haplotype to foresee warning signs such as minor symptoms. In diseases such as prostate cancer, in which metastasis can be fatal if not stopped in time, the knowledge of a potential predisposition, might contribute in a very significant manner to treatment efficacy. Similarly, a diagnosed predisposition to a potential side-effect could immediately direct the physician toward a treatment for which such side-effects have not been observed during clinical trials.

The invention concerns a method for the detection in an individual of alleles of *PCTA-1*-related biallelic markers associated with a trait preferably selected from prostate cancer, an early onset of prostate cancer, a susceptibility to prostate cancer, the level of aggressiveness of prostate cancer tumors, or the level of expression of the *PCTA-1* gene. The information obtained using this method is useful in the diagnosis, staging, monitoring, prognosis and/or prophylactic or curative therapy of prostate cancer. The method also concerns the detection of

specific alleles present within the *PCTA-1* gene expressing a modified level of *PCTA-1* mRNA or an altered *PCTA-1* mRNA, coding for an altered PCTA-1 protein. The identities of the polymorphic bases may be determined using any of the genotyping procedures described above in "Method For Genotyping An Individual For Biallelic Markers". More particularly, the invention concerns the detection of a *PCTA-1* nucleic acid comprising at least one of the nucleotide sequences of P1 to P125 and the complementary sequence thereof. This method comprises the following steps:

- obtaining a nucleic acid sample from the individual to be tested; and

- determining the presence in the sample of an allele of a biallelic marker or of a group of biallelic markers of the *PCTA-1* gene which, when taken alone or in combination with another/other biallelic marker/s of the *PCTA-1* gene, is indicative of prostate cancer, of an early onset of prostate cancer, of the level of aggressiveness of prostate cancer tumors, of a modified or forthcoming expression of the *PCTA-1* gene, of a modified or forthcoming production of the PCTA-1 protein, or of the production of a modified PCTA-1 protein.

In some embodiments, the biallelic marker comprises at least one of the biallelic markers defined by the sequences P1 to P125, and the complementary sequences thereto, more preferably at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In a preferred embodiment, the biallelic marker comprises at least one of the biallelic markers defined by the sequences of P2, P30, P41, P55, P57, and the complementary sequence thereto, more particularly at least one biallelic marker selected from the group consisting of A2, A30, A41, A55, A57 and the complement thereof. In a preferred embodiment, the detection method comprises an additional step of amplifying a nucleotide sequence of the *PCTA-1* gene comprising biallelic markers. Optionally, the amplification primers can be selected from the group consisting of B1 to B47 and C1 to C47.

In preferred embodiments of the detection method described above, the presence of alleles of one or more biallelic markers of the *PCTA-1* gene is determined through microsequencing reactions. Optionally, the microsequencing primers are selected from the group consisting of D1 to D125 and E1 to E125. Optionally, the microsequencing primers can be bound to a solid support, preferably in the form of arrays of primers attached to appropriate chips or be used in microfluidic devices. Such arrays are described in further detail in the "Oligonucleotide arrays" section. Optionally, the microsequencing primers can be labeled.

In additional preferred embodiments of the detection method, the presence of alleles of one or more biallelic markers of the *PCTA-1* gene is determined through an allele specific amplification assay or an enzyme based mismatch detection assay. Optionally, the allele

specific amplification assay comprises a step of detecting the presence of the amplification product.

In further preferred embodiments of the detection method, the presence of alleles of one or more biallelic markers of the *PCTA-1* gene is determined through a hybridization assay. The probes used in the hybridization assay may include a probe selected from the group consisting of P1 to P125, a complementary sequence thereto or a fragment thereof, said fragment comprising the polymorphic base. Preferably, the probe is labeled.

A diagnostic method according to the present invention can also consist on the detection of an allele of the *PCTA-1* gene comprising a trait causing mutation.

The invention also specifically relates to a method of determining whether an individual suffering from prostate cancer or susceptible of developing prostate cancer is likely to respond positively to treatment with a selected medicament acting against prostate cancer.

The method comprises the following steps:

- obtaining a DNA sample from the individual to be tested; and
- analyzing said DNA sample to determine whether it comprises alleles of one or more biallelic markers associated with a positive response to treatment with the medicament and/or alleles of one or more biallelic markers associated with a negative response to treatment with the medicament.

In a preferred embodiment, the biallelic marker is selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith.

The detection methods of the present invention can be applied to, for example, the preliminary screening of patient populations suffering from prostate cancer. This preliminary screening is useful to initiate adequate treatment when needed or to determine and select appropriate patient populations for clinical trials on new compounds in order to avoid the potential occurrence of specific side effects or to enhance the probability of beneficial patient response. By establishing in advance a homogeneous genotype selection for the population to be tested, the assessment of drug efficacy and/or toxicity can be more readily achieved and less hampered by divergences in population response. This approach can yield better therapeutic approaches based on patient population targeting resulting from pharmacogenomics studies.

The invention also relates to diagnostic kits useful for determining the presence in a DNA sample of alleles associated with the trait, preferably with prostate cancer, with an early onset of prostate cancer, with the level of aggressiveness of prostate cancer tumors, with a modified or forthcoming expression of the *PCTA-1* gene, with a modified or forthcoming

production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein. Diagnostic kits can comprise any of the polynucleotides of the present invention.

In a first embodiment, the kit comprises primers such as those described above, preferably forward and reverse primers which are used to amplify the *PCTA-1* gene or a fragment thereof. In some embodiments, at least one of the primers is complementary to a nucleotide sequence of the *PCTA-1* gene comprising a biallelic marker associated with prostate cancer, with an early onset of prostate cancer, with the level of aggressiveness of prostate cancer tumors, with a modified or forthcoming expression of the *PCTA-1* gene, with a modified or forthcoming production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein. In one embodiment, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Optionally, the kit comprises an amplification primer which includes a polymorphic base of at least one biallelic marker selected from the group consisting of A1 to A125 and the complements thereof. In a preferred embodiment, the kit comprises one or more of the sequences B1 to B47 and C1 to C47. In a more preferred embodiment, the kit comprises one or more of the sequences B1, B16, B20, B23, B24 and C1, C16, C20, C23, C24.

In a second embodiment, the kit comprises microsequencing primers, wherein at least one of said primers is an oligonucleotide capable of hybridizing, either with the coding or with the non-coding strand, immediately upstream of the polymorphic base of a biallelic marker selected from the group consisting of A1 to A125 and the complements thereof, preferably those of D1 to D125 and E1 to E125, more preferably those of D2, D30, D41, D55, D57 and E2, E30, E41, E55, E57.

In a third embodiment, the kit comprises a hybridization DNA probe, that is or eventually becomes immobilized on a solid support, which is capable of hybridizing with the *PCTA-1* gene or fragment thereof, preferably which is capable of hybridizing with a region of the *PCTA-1* gene which comprises an allele of a biallelic marker of the present invention, more preferably an allele associated with prostate cancer, with an early onset of prostate cancer, with a susceptibility to prostate cancer, with the level of aggressiveness of prostate cancer tumors, with a modified or forthcoming expression of the *PCTA-1* gene, with a modified or forthcoming production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein. In a preferred embodiment, the probe is selected from the group consisting of P1 to P125 and the complementary sequences thereto, or a fragment thereof, said fragment comprising the polymorphic base. In a more preferred embodiment, the probe is selected from the group consisting of P2, P30, P41, P55, P57 and the complementary sequences thereto, or a fragment thereof, said fragment comprising the polymorphic base.

The kits of the present invention can also comprise optional elements including appropriate amplification reagents such as DNA polymerases when the kit comprises primers, reagents useful in hybridization reactions and reagents useful to reveal the presence of a hybridization reaction between a labeled hybridization probe and the *PCTA-1* gene containing at least one biallelic marker.

Treatment Of Cancer or Prostate Cancer

The invention also concerns methods for the treatment of prostate cancer using an allele of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with a susceptibility to prostate cancer, with an aggressive form of prostate cancer or with a positive or negative response to treatment with an effective amount of a medicament acting against prostate cancer.

As the metastasis of prostate cancer can be fatal, it is important to detect prostate cancer susceptibility of individuals. Consequently, the invention also concerns a method for the treatment of prostate cancer comprising the following steps:

- selecting an individual whose DNA comprises alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with prostate cancer;

- following up said individual for the appearance (and optionally the development) of tumors in prostate; and

- administering an effective amount of a medicament acting against prostate cancer to said individual at an appropriate stage of the prostate cancer.

In some embodiments, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In particular embodiments, the individual is selected by genotyping one or more biallelic markers of the present invention.

The prophylactic administration of a treatment serves to prevent, attenuate or inhibit the growth of cancer cells.

Therefore, another embodiment of the present invention consists of a method for the treatment of prostate cancer comprising the following steps:

- selecting an individual whose DNA comprises alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with prostate cancer; and

- administering to said individual, preferably as a preventive treatment of prostate cancer, an effective amount of a medicament acting against prostate cancer such as 4HPR or of a vaccine composition capable of conferring immunity against *PCTA-1* related prostate cancer.

In some embodiments, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. More preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A2, A30, A41, A55, A57, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In particular embodiments, the individual is selected by genotyping one or more biallelic markers of the present invention.

In a further embodiment, the present invention concerns a method for the treatment of prostate cancer comprising the following steps:

- selecting an individual whose DNA comprises alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with a susceptibility prostate cancer;

- administering to said individual, as a preventive treatment of prostate cancer, an effective amount of a medicament acting against prostate cancer such as 4HPR or of a vaccine composition capable of conferring immunity against *PCTA-1*-related prostate cancer;

- following up said individual for the appearance and the development of tumors in prostate; and optionally

- administering an effective amount of a medicament acting against prostate cancer to said individual at the appropriate stage of the prostate cancer.

In some embodiments, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. More preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A2, A30, A41, A55, A57, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In particular embodiments, the individual is selected by genotyping one or more biallelic markers of the present invention.

To enlighten the choice of the appropriate beginning of the treatment of prostate cancer, the present invention also concerns a method for the treatment of prostate cancer comprising the following steps:

- selecting an individual suffering from a prostate cancer whose DNA comprises alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with the aggressiveness of prostate cancer tumors; and

- administering an effective amount of a medicament acting against prostate cancer to said individual.

In some embodiments, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. More preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A2, A30, A41, A55, A57, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In particular embodiments, the individual is selected by genotyping one or more biallelic markers of the present invention.

The invention concerns a method of determining whether a subject is likely to respond positively to treatment with a selected medicament acting against prostate cancer.

The invention also concerns a method for the treatment of prostate cancer in a selected population of individuals. The method comprises :

- selecting an individual suffering from prostate cancer and

- whose DNA comprises alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with a positive response to treatment with an effective amount of a medicament acting against prostate cancer,

- and/or whose DNA does not comprise alleles of a biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with a negative response to treatment with said medicament; and

- administering at suitable intervals an effective amount of said medicament to said selected individual.

In some embodiments, the biallelic marker is comprised in one of the sequences P1 to P125 and the complementary sequences thereto. Preferably the biallelic marker is at least one biallelic marker selected from the group consisting of A1 to A125, and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In particular embodiments, the individual is selected by genotyping one or more biallelic markers of the present invention.

Another aspect of the invention is a method of using a medicament acting against prostate cancer. The method comprises obtaining a DNA sample from a subject, determining whether the DNA sample contains one or more biallelic markers associated with a positive

response to the medicament and/or whether the DNA sample contains one or more biallelic markers associated with a negative response to the medicament, and administering the medicament to the subject if the DNA sample contains one or more biallelic markers associated with a positive response to the medicament and/or if the DNA sample lacks one or more biallelic markers associated with a negative response to the medicament.

The invention also concerns a method for the clinical testing of a medicament, preferably a medicament acting against prostate cancer.

In some embodiments, the medicament may be administered to the subject in a clinical trial if the DNA sample contains alleles of one or more biallelic markers associated with a positive response to treatment with the medicament and/or if the DNA sample lacks alleles of one or more biallelic markers associated with a negative response to treatment with the medicament. In preferred embodiments, the medicament is a drug acting against prostate cancer. In other embodiments, the biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof or optionally the biallelic markers in linkage disequilibrium therewith.

Using the method of the present invention, the evaluation of drug efficacy may be conducted in a population of individuals likely to respond favorably to the medicament.

The invention also concerns a method for the clinical testing of a medicament, preferably a medicament acting against prostate cancer. The method comprises the following steps:

- administering a medicament, preferably a medicament susceptible of acting against prostate cancer to a heterogeneous population of individuals;
- identifying a first population of individuals who respond positively to said medicament and a second population of individuals who respond negatively to said medicament;
- identifying biallelic markers in said first population which are associated with a positive response to said medicament;
- selecting individuals whose DNA comprises biallelic markers associated with a positive response to said medicament; and
- administering said medicament to said individuals.

Such methods are deemed to be extremely useful to increase the benefit/risk ratio resulting from the administration of medicaments which may cause undesirable side effects and/or be inefficacious to a portion of the patient population to which it is normally administered.

Once an individual has been diagnosed as suffering from a prostate cancer, selection tests are carried out to determine whether the DNA of this individual comprises alleles of a biallelic marker or of a group of biallelic markers associated with a positive response to treatment or with a negative response to treatment which may include either side effects or unresponsiveness.

The selection of the patient to be treated using the method of the present invention can be carried out through the detection methods described above. The individuals which are to be selected are preferably those whose DNA does not comprise alleles of a biallelic marker or of a group of biallelic markers associated with a negative response to treatment. The knowledge of an individual's genetic predisposition to unresponsiveness or side effects to particular medicaments allows the clinician to direct treatment toward appropriate drugs against prostate cancer.

Once the patient's genetic predispositions have been determined, the clinician can select appropriate treatment for which negative response, particularly side effects, has not been reported or has been reported only marginally for the patient.

Recombinant Vectors

The term "vector" is used herein to designate either a circular or a linear DNA or RNA molecule, which is either double-stranded or single-stranded, and which comprise at least one polynucleotide of interest that is sought to be transferred in a cell host or in a unicellular or multicellular host organism.

Another embodiment of the present invention is a recombinant vector. This recombinant vector comprises a nucleotide sequence encoding a regulatory region of the *PCTA-1* gene, the promoter region of the *PCTA-1* gene, an intron of the *PCTA-1* gene, exon 0 and/or exon 1 of the *PCTA-1* gene, exon 6bis of the *PCTA-1* gene, exon 9bis of the *PCTA-1* gene, the genomic sequence of the *PCTA-1* gene, a cDNA sequence of the *PCTA-1* gene, or combinations of such sequences, or complementary sequences thereto or fragments or variants thereof. Preferred nucleotide sequences included in such an expression vector include at least one nucleotide sequence selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 8 or fragments or variants thereof or a complementary sequence thereto.

Generally, a recombinant vector of the invention may comprise any of the polynucleotides described herein, including regulatory sequences and coding sequences, as well as any *PCTA-1* primer or probe as defined above. More particularly, the recombinant vectors of the present invention can comprise any of the polynucleotides described in the "*PCTA-1* cDNA

Sequences" section, the "Coding Regions" section, and the "Oligonucleotide Probes And Primers" section.

In another embodiment, the vector includes a *PCTA-1* gene or cDNA or a fragment thereof comprising at least one of the biallelic markers described herein, and more preferably a mutated *PCTA-1* gene or cDNA comprising a trait causing mutation, particularly a mutation determined using the method described above. Preferably, the biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith.

One embodiment of the invention is the production of a PCTA-1 protein under the control of its own promoter or of an exogenous promoter. The present invention also relates to expression vectors which include nucleic acids encoding a native or mutated PCTA-1 protein under the control of either a native *PCTA-1* regulatory region, preferably a native *PCTA-1* promoter which comprises at least one of the biallelic markers of the present invention, more particularly at least one among the A1 to A43 and the complements thereof, or an exogenous promoter.

More particularly, the present invention relates to expression vectors which include nucleic acids encoding a PCTA-1 protein, preferably a *PCTA-1* protein comprising a amino acid sequence selected from the group consisting of SEQ ID Nos 5, 6, 7, 9 or variants or fragments thereof, under the control of a regulatory sequence selected among the *PCTA-1* regulatory polynucleotides, or alternatively under the control of an exogenous regulatory sequence.

The present invention also concerns an expression vector comprising a *PCTA-1* regulatory region or any sequence thereof of 10 to 3000 nucleotides capable of regulating the expression of a nucleotide sequence encoding a protein and operably linked to the regulatory region. A further preferred regulatory region is the promoter sequence. In this regard, it is to be noted that a portion of the promoter can be used in the expression vector as long as it can influence the transcription of the coding sequence operably linked thereto.

Any nucleotide sequence encoding a polypeptide of interest can be included in an expression vector comprising a *PCTA-1* regulatory region and operably linked thereto. Preferred polypeptides are therapeutic proteins which are described in further detail later on.

In some embodiments, expression vectors are employed to express a *PCTA-1* polypeptide which can be then purified and, for example be used in ligand screening assays or as an immunogen in order to raise specific antibodies directed against a *PCTA-1* protein. In other embodiments, the expression vectors are used for constructing transgenic animals and also for gene therapy.

Some of the elements which can be found in the vectors of the present invention are described in further detail in the following sections.

1. General features of the expression vectors of the invention

A recombinant vector according to the invention comprises, but is not limited to, a YAC (Yeast Artificial Chromosome), a BAC (Bacterial Artificial Chromosome), a phage, a phagemid, a cosmid, a plasmid or even a linear DNA molecule which may consist of a chromosomal, non-chromosomal, semi-synthetic or synthetic DNA. Such a recombinant vector can comprise a transcriptional unit comprising an assembly of:

(1) a genetic element or elements having a regulatory role in gene expression, for example promoters or enhancers. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp in length that act on the promoter to increase the transcription.

(2) a structural or coding sequence which is transcribed into mRNA and eventually translated into a polypeptide, said structural or coding sequence being operably linked to the regulatory elements described in (1); and

(3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, when a recombinant protein is expressed without a leader or transport sequence, it may include a N-terminal residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

Generally, recombinant expression vectors will include origins of replication, selectable markers permitting transformation of the host cell, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably a leader sequence capable of directing secretion of the translated protein into the periplasmic space or the extracellular medium. In a specific embodiment wherein the vector is adapted for transfecting and expressing desired sequences in mammalian host cells, preferred vectors will comprise an origin of replication in the desired host, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking non-transcribed sequences. DNA sequences derived from the SV40 viral genome, for example SV40 origin, early promoter, enhancer, splice and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

The *in vivo* expression of a PCTA-1 polypeptide may be useful in order to correct a genetic defect related to the expression of the native gene in a host organism or to the production of a biologically inactive PCTA-1 protein.

Consequently, the present invention also deals with recombinant expression vectors mainly designed for the *in vivo* production of a PCTA-1 polypeptide of SEQ ID Nos 5, 6, 7, 9 or fragments or variants thereof by the introduction of the appropriate genetic material in the organism of the patient to be treated. This genetic material may be introduced *in vitro* in a cell that has been previously extracted from the organism, the modified cell being subsequently reintroduced in the said organism, directly *in vivo* into the appropriate tissue.

2. Regulatory Elements

Promoters

The suitable promoter regions used in the expression vectors according to the present invention are chosen taking into account the cell host in which the heterologous gene has to be expressed. The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell, such as, for example, a human or a viral promoter.

A suitable promoter may be heterologous with respect to the nucleic acid for which it controls the expression or alternatively can be endogenous to the native polynucleotide containing the coding sequence to be expressed. Additionally, the promoter is generally heterologous with respect to the recombinant vector sequences within which the construct promoter/coding sequence has been inserted.

Promoter regions can be selected from any desired gene using, for example, CAT (chloramphenicol transferase) vectors and more preferably pKK232-8 and pCM7 vectors.

Preferred bacterial promoters are the LacI, LacZ, the T3 or T7 bacteriophage RNA polymerase promoters, the gpt, lambda PR, PL and trp promoters (EP 0036776, the disclosure of which is incorporated herein by reference in its entirety), the polyhedrin promoter, or the p10 protein promoter from baculovirus (Kit Novagen) (Smith et al., 1983; O'Reilly et al., 1992), the lambda PR promoter or also the trc promoter.

Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionine-L. Selection of a convenient vector and promoter is well within the level of ordinary skill in the art.

The choice of a promoter is well within the ability of a person skilled in the field of genetic engineering. For example, one may refer to the book of Sambrook et al.(1989) or also to the procedures described by Fuller et al.(1996).

Other Regulatory Elements

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

The vector containing the appropriate DNA sequence as described above, more preferably *PCTA-1* gene regulatory polynucleotide, a polynucleotide encoding a PCTA-1 polypeptide selected from the group consisting of SEQ ID No 1 or a fragment or a variant thereof and SEQ ID Nos 2, 3, 4, 8, or both of them, can be utilized to transform an appropriate host to allow the expression of the desired polypeptide or polynucleotide.

3. Selectable Markers

Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. The selectable marker genes for selection of transformed host cells are preferably dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, TRP1 for *S. cerevisiae* or tetracycline, rifampicin or ampicillin resistance in *E. coli*, or levan saccharase for mycobacteria, this latter marker being a negative selection marker.

4. Preferred Vectors.

Bacterial Vectors

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and a bacterial origin of replication derived from commercially available plasmids comprising genetic elements of pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia, Uppsala, Sweden), and GEM1 (Promega Biotec, Madison, WI, USA).

Large numbers of other suitable vectors are known to those of skill in the art, and commercially available, such as the following bacterial vectors: pQE70, pQE60, pQE-9

(Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pMSG, pSVL (Pharmacia); pQE-30 (QIAexpress).

Bacteriophage Vectors

The P1 bacteriophage vector may contain large inserts ranging from about 80 to about 100 kb.

The construction of P1 bacteriophage vectors such as p158 or p158/neo8 are notably described by Sternberg (1994). Recombinant P1 clones comprising *PCTA-1* nucleotide sequences may be designed for inserting large polynucleotides of more than 40 kb (Linton et al., 1993). To generate P1 DNA for transgenic experiments, a preferred protocol is the protocol described by McCormick et al.(1994). Briefly, *E. coli* (preferably strain NS3529) harboring the P1 plasmid are grown overnight in a suitable broth medium containing 25 μg/ml of kanamycin. The P1 DNA is prepared from the *E. coli* by alkaline lysis using the Qiagen Plasmid Maxi kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. The P1 DNA is purified from the bacterial lysate on two Qiagen-tip 500 columns, using the washing and elution buffers contained in the kit. A phenol/chloroform extraction is then performed before precipitating the DNA with 70% ethanol. After solubilizing the DNA in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), the concentration of the DNA is assessed by spectrophotometry.

When the goal is to express a P1 clone comprising *PCTA-1* nucleotide sequences in a transgenic animal, typically in transgenic mice, it is desirable to remove vector sequences from the P1 DNA fragment, for example by cleaving the P1 DNA at rare-cutting sites within the P1 polylinker (*Sfi*I, *Not*I or *Sal*I). The P1 insert is then purified from vector sequences on a pulsed-field agarose gel, using methods similar using methods similar to those originally reported for the isolation of DNA from YACs (Schedl et al., 1993a; Peterson et al., 1993). At this stage, the resulting purified insert DNA can be concentrated, if necessary, on a Millipore Ultrafree-MC Filter Unit (Millipore, Bedford, MA, USA – 30,000 molecular weight limit) and then dialyzed against microinjection buffer (10 mM Tris-HCl, pH 7.4; 250 μM EDTA) containing 100 mM NaCl, 30 μM spermine, 70 μM spermidine on a microdyalisis membrane (type VS, 0.025 μM from Millipore). The intactness of the purified P1 DNA insert is assessed by electrophoresis on 1% agarose (Sea Kem GTG; FMC Bio-products) pulse-field gel and staining with ethidium bromide.

Baculovirus Vectors

A suitable vector for the expression of a PCTA-1 polypeptide of SEQ ID Nos 5, 6, 7, 9 or fragments or variants thereof is a baculovirus vector that can be propagated in insect cells and in insect cell lines. A specific suitable host vector system is the pVL1392/1393 baculovirus transfer vector (Pharmingen) that is used to transfect the SF9 cell line (ATCC N°CRL 1711) which is derived from *Spodoptera frugiperda.*

Other suitable vectors for the expression of a PCTA-1 polypeptide of SEQ ID Nos 5, 6, 7, 9 or fragments or variants thereof in a baculovirus expression system include those described by Chai et al.(1993), Vlasak et al.(1983) and Lenhard et al.(1996).

Viral Vectors

In one specific embodiment, the vector is derived from an adenovirus. Preferred adenovirus vectors according to the invention are those described by Feldman and Steg (1996) or Ohno et al.(1994). Another preferred recombinant adenovirus according to this specific embodiment of the present invention is the human adenovirus type 2 or 5 (Ad 2 or Ad 5) or an adenovirus of animal origin (French patent application N° FR-93.05954).

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery systems of choice for the transfer of exogenous polynucleotides *in vivo* , particularly to mammals, including humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host.

Particularly preferred retroviruses for the preparation or construction of retroviral *in vitro* or *in vitro* gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Mink-Cell Focus Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma virus. Particularly preferred Murine Leukemia Viruses include the 4070A and the 1504A viruses, Abelson (ATCC No VR-999), Friend (ATCC No VR-245), Gross (ATCC No VR-590), Rauscher (ATCC No VR-998) and Moloney Murine Leukemia Virus (ATCC No VR-190; PCT Application No WO 94/24298). Particularly preferred Rous Sarcoma Viruses include Bryan high titer (ATCC Nos VR-334, VR-657, VR-726, VR-659 and VR-728). Other preferred retroviral vectors are those described in Roth et al.(1996), PCT Application No WO 93/25234 (the disclosure of which is incorporated herein by reference in its entirety), PCT Application No WO 94/ 06920 (the disclosure of which is incorporated herein by reference in its entirety), Roux et al., 1989, Julan et al., 1992 and Neda et al., 1991.

Yet another viral vector system that is contemplated by the invention consists of the adeno-associated virus (AAV). The adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka et al., 1992). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (Flotte et al., 1992; Samulski et al., 1989; McLaughlin et al., 1989). One advantageous feature of AAV derives from its reduced efficacy for transducing primary cells relative to transformed cells.

BAC Vectors

The bacterial artificial chromosome (BAC) cloning system (Shizuya et al., 1992) has been developed to stably maintain large fragments of genomic DNA (100-300 kb) in *E. coli*. A preferred BAC vector consists of pBeloBAC11 vector that has been described by Kim et al.(1996). BAC libraries are prepared with this vector using size-selected genomic DNA that has been partially digested using enzymes that permit ligation into either the *Bam* HI or *Hind*III sites in the vector. Flanking these cloning sites are T7 and SP6 RNA polymerase transcription initiation sites that can be used to generate end probes by either RNA transcription or PCR methods. After the construction of a BAC library in *E. coli*, BAC DNA is purified from the host cell as a supercoiled circle. Converting these circular molecules into a linear form precedes both size determination and introduction of the BACs into recipient cells. The cloning site is flanked by two *Not* I sites, permitting cloned segments to be excised from the vector by *Not* I digestion. Alternatively, the DNA insert contained in the pBeloBAC11 vector may be linearized by treatment of the BAC vector with the commercially available enzyme lambda terminase that leads to the cleavage at the unique *cos*N site, but this cleavage method results in a full length BAC clone containing both the insert DNA and the BAC sequences.

5. Delivery Of The Recombinant Vectors

In order to effect expression of the polynucleotides and polynucleotide constructs of the invention, these constructs must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cell lines, or *in vivo* or *ex vivo*, as in the treatment of certain diseases states.

One mechanism is viral infection where the expression construct is encapsulated in an infectious viral particle.

Several non-viral methods for the transfer of polynucleotides into cultured mammalian cells are also contemplated by the present invention, and include, without being limited to, calcium phosphate precipitation (Graham et al., 1973; Chen et al., 1987;), DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa et al., 1986; Potter et al., 1984), direct microinjection

(Harland et al., 1985), DNA-loaded liposomes (Nicolau et al., 1982; Fraley et al., 1979), and receptor-mediate transfection (Wu and Wu, 1987; 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression polynucleotide has been delivered into the cell, it may be stably integrated into the genome of the recipient cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle.

One specific embodiment for a method for delivering a protein or peptide to the interior of a cell of a vertebrate *in vivo* comprises the step of introducing a preparation comprising a physiologically acceptable carrier and a naked polynucleotide operatively coding for the polypeptide of interest into the interstitial space of a tissue comprising the cell, whereby the naked polynucleotide is taken up into the interior of the cell and has a physiological effect. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* as well.

Compositions for use *in vitro* and *in vivo* comprising a "naked" polynucleotide are described in PCT application N° WO 90/11092 (Vical Inc.) and also in PCT application No. WO 95/11307 (Institut Pasteur, INSERM, Université d'Ottawa) as well as in the articles of Tacson et al.(1996) and of Huygen et al.(1996).

In still another embodiment of the invention, the transfer of a naked polynucleotide of the invention, including a polynucleotide construct of the invention, into cells may be proceeded with a particle bombardment (biolistic), said particles being DNA-coated microprojectiles accelerated to a high velocity allowing them to pierce cell membranes and enter cells without killing them, such as described by Klein et al.(1987).

In a further embodiment, the polynucleotide of the invention may be entrapped in a liposome (Ghosh and Bacchawat, 1991; Wong et al., 1980; Nicolau et al., 1987)

In a specific embodiment, the invention provides a composition for the *in vivo* production of a PCTA-1 protein or polypeptide described herein. It comprises a naked polynucleotide operatively coding for this polypeptide, in solution in a physiologically acceptable carrier, and suitable for introduction into a tissue to cause cells of the tissue to express the said protein or polypeptide.

The amount of vector to be injected to the desired host organism varies according to the site of injection. As an indicative dose, it will be injected between 0,1 and 100 µg of the vector in an animal body, preferably a mammal body, for example a mouse body.

In another embodiment of the vector according to the invention, it may be introduced *in vitro* in a host cell, preferably in a host cell previously harvested from the animal to be treated and more preferably a somatic cell such as a muscle cell. In a subsequent step, the cell that has been transformed with the vector coding for the desired PCTA-1 polypeptide or the desired fragment thereof is reintroduced into the animal body in order to deliver the recombinant protein within the body either locally or systemically.

Cell Hosts

The invention also concerns host cells transformed by one of the vectors described above that produce either a heterologous protein, a PCTA-1 protein or fragments thereof encoded by the *PCTA-1* gene, preferably comprising at least one of the biallelic polymorphisms described herein, and more preferably a mutated *PCTA-1* gene comprising the trait causing mutation determined using the above-noted method.

Another object of the invention consists of a host cell that has been transformed or transfected with one of the polynucleotides described herein, and in particular a polynucleotide either comprising a *PCTA-1* regulatory polynucleotide or the coding sequence of a PCTA-1 polypeptide selected from the group consisting of SEQ ID No 1 2, 3, 4, 8 or a fragment or a variant thereof. Also included are host cells that are transformed (prokaryotic cells) or that are transfected (eukaryotic cells) with a recombinant vector such as one of those described above. More particularly, the cell hosts of the present invention can comprise any of the polynucleotides described in the "*PCTA-1* cDNA Sequences" section, the "Coding Regions" section, and the "Oligonucleotide Probes And Primers" section.

A further recombinant cell host according to the invention comprises a polynucleotide containing a biallelic marker selected from the group consisting of A1 to A125, and the complements thereof.

Generally, a recombinant host cell of the invention comprises any one of the polynucleotides or the recombinant vectors described herein.

Preferred host cells used as recipients for the expression vectors of the invention are the following:

a) Prokaryotic host cells: *Escherichia coli* strains (I.E.DH5-α strain), *Bacillus subtilis*, *Salmonella typhimurium*, and strains from species like *Pseudomonas*, *Streptomyces* and *Staphylococcus*.

b) Eukaryotic host cells: HeLa cells (ATCC N°CCL2; N°CCL2.1; N°CCL2.2), Cv 1 cells (ATCC N°CCL70), COS cells (ATCC N°CRL1650; N°CRL1651), Sf-9 cells (ATCC N°CRL1711), C127 cells (ATCC N° CRL-1804), 3T3 (ATCC N° CRL-6361), CHO (ATCC N°

CCL-61), human kidney 293. (ATCC N° 45504; N° CRL-1573) and BHK (ECACC N° 84100501; N° 84111301).

c) Other mammalian host cells.

The *PCTA-1* gene expression in mammalian, and typically human, cells may be rendered defective, or alternatively it may be proceeded with the insertion of a *PCTA-1* genomic or cDNA sequence with the replacement of the *PCTA-1* gene counterpart in the genome of an animal cell by a *PCTA-1* polynucleotide according to the invention. These genetic alterations may be generated by homologous recombination events using specific DNA constructs that have been previously described.

One kind of cell hosts that may be used are mammal zygotes, such as murine zygotes. For example, murine zygotes may undergo microinjection with a purified DNA molecule of interest, for example a purified DNA molecule that has previously been adjusted to a concentration range from 1 ng/ml –for BAC inserts- 3 ng/µl –for P1 bacteriophage inserts- in 10 mM Tris-HCl, pH 7.4, 250 µM EDTA containing 100 mM NaCl, 30 µM spermine, and70 µM spermidine. When the DNA to be microinjected has a large size, polyamines and high salt concentrations can be used in order to avoid mechanical breakage of this DNA, as described by Schedl et al (1993b).

Anyone of the polynucleotides of the invention, including the DNA constructs described herein, may be introduced in an embryonic stem (ES) cell line, preferably a mouse ES cell line. ES cell lines are derived from pluripotent, uncommitted cells of the inner cell mass of pre-implantation blastocysts. Preferred ES cell lines are the following: ES-E14TG2a (ATCC n° CRL-1821), ES-D3 (ATCC n° CRL1934 and n° CRL-11632), YS001 (ATCC n° CRL-11776), 36.5 (ATCC n° CRL-11116). To maintain ES cells in an uncommitted state, they are cultured in the presence of growth inhibited feeder cells which provide the appropriate signals to preserve this embryonic phenotype and serve as a matrix for ES cell adherence. Preferred feeder cells consist of primary embryonic fibroblasts that are established from tissue of day 13-day 14 embryos of virtually any mouse strain, that are maintained in culture, such as described by Abbondanzo et al.(1993) and are inhibited in growth by irradiation, such as described by Robertson (1987), or by the presence of an inhibitory concentration of LIF, such as described by Pease and Williams (1990).

The constructs in the host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence.

Following transformation of a suitable host and growth of the host to an appropriate cell density, the selected promoter is induced by appropriate means, such as temperature shift or chemical induction, and cells are cultivated for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in the expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known by the skill artisan.

Transgenic Animals

The invention also relates to transgenic animals having an exogenous *PCTA-1* regulatory region or a *PCTA-1* gene, preferably comprising at least one of the biallelic polymorphisms described herein, and more preferably to a mutated *PCTA-1* gene comprising the trait causing mutation determined using the above-noted method. Preferably, the biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof, or optionally the biallelic markers in linkage disequilibrium therewith. In another embodiment, the invention concerns animals, preferably a mouse, having the mouse *PCTA-1* gene which is modified or knocked out. These animals could be used to screen compounds of interest.

The terms "transgenic animals" or "host animals" are used herein to designate animals that have their genome genetically and artificially manipulated so as to include one of the nucleic acids according to the invention. Preferred animals are non-human mammals and include those belonging to a genus selected from Mus (e.g. mice), Rattus (e.g. rats) and Oryctogalus (e.g. rabbits) which have their genome artificially and genetically altered by the insertion of a nucleic acid according to the invention.

In one embodiment, the invention encompasses non-human host mammals and animals comprising a recombinant vector of the invention, a polynucleotide construct according to the invention, or a *PCTA-1* gene disrupted by homologous recombination with a knock out vector. Generally, a transgenic animal according the present invention comprises any one of the polynucleotides, the recombinant vectors and the cell hosts described in the present invention. More particularly, the transgenic animals according to the present invention can comprise any of the polynucleotides described in the "*PCTA-1* cDNA Sequences" section, the "Coding Regions" section, and the "Oligonucleotide Probes And Primers" section.

The transgenic animals of the invention all include within a plurality of their cells a cloned recombinant or synthetic DNA sequence, more specifically one of the purified or isolated nucleic acids comprising a *PCTA-1* coding sequence, a *PCTA-1* regulatory polynucleotide or a DNA sequence encoding an antisense polynucleotide such as described in the present specification, and still more preferably a nucleotide comprising an allele of at least one biallelic marker of the *PCTA-1* gene.

In a first preferred embodiment, these transgenic animals may be good experimental models in order to study cancer, preferably prostate cancer, in particular concerning the transgenic animals within the genome of which has been inserted one or several copies of a polynucleotide encoding a native PCTA-1 protein, or alternatively a mutant PCTA-1 protein.

In a second preferred embodiment, these transgenic animals may express a desired polypeptide of interest under the control of the regulatory polynucleotides of the *PCTA-1* gene, leading to good yields in the synthesis of this protein of interest, and eventually a tissue specific expression of this protein of interest.

The design of the transgenic animals of the invention may be made according to the conventional techniques well known from the one skilled in the art. For more details regarding the production of transgenic animals, and specifically transgenic mice, it may be referred to US Patents Nos 4,873,191, issued Oct. 10, 1989; 5,464,764 issued Nov 7, 1995; and 5,789,215, issued Aug 4, 1998, the disclosures of which are incorporated herein by reference in their entireties.

Transgenic animals of the present invention are produced by the application of procedures which result in an animal with a genome that incorporates exogenous genetic material which is integrated into the genome. The procedure involves obtaining the genetic material, or a portion thereof, which encodes either a *PCTA-1* coding sequence, a *PCTA-1* regulatory polynucleotide or a DNA sequence encoding an antisense polynucleotide such as described in the present specification.

A recombinant polynucleotide of the invention is inserted into an embryonic or ES stem cell line. The insertion is made using electroporation. The cells subjected to electroporation are screened (e.g. Southern blot analysis) to find positive cells which have integrated the exogenous recombinant polynucleotide into their genome. An illustrative positive-negative selection procedure that may be used according to the invention is described by Mansour et al. (1988).

Then, the positive cells are isolated, cloned and injected into 3.5 days old blastocysts from mice. The blastocysts are then inserted into a female host animal and allowed to grow to term.

Alternatively, the positive ES cells are brought into contact with embryos at the 2.5 days old 8-16 cell stage (morulae) such as described by Wood et al.(1993) or by Nagy et al.(1993), the ES cells being internalized to colonize extensively the blastocyst including the cells which will give rise to the germ line.

The offsprings of the female host are tested to determine which animals are transgenic e.g. include the inserted exogenous DNA sequence and which are wild-type.

Thus, the present invention also concerns a transgenic animal containing a nucleic acid, a recombinant expression vector or a recombinant host cell according to the invention.

Recombinant Cell Lines Derived From The Transgenic Animals Of The Invention.

A further object of the invention consists of recombinant host cells obtained from a transgenic animal described herein. In one embodiment the invention encompasses cells derived from non-human host mammals and animals comprising a recombinant vector of the invention or a *PCTA-1* gene disrupted by homologous recombination with a knock out vector.

Recombinant cell lines may be established *in vitro* from cells obtained from any tissue of a transgenic animal according to the invention, for example by transfection of primary cell cultures with vectors expressing *onc*-genes such as SV40 large T antigen, as described by Chou (1989) and Shay et al.(1991).

Screening Of Agents Acting Against Prostate Cancer

In a further embodiment, the present invention also concerns a method for the screening of new agents, or candidate substances, acting against cancer, preferably against prostate cancer and which may be suitable for the treatment of a patient whose DNA comprises an allele of the PCTA-1 gene associated with cancer, preferably with prostate cancer, with an early onset of prostate cancer, or with the aggressiveness of prostate cancer tumors, or more generally with a modified or forthcoming expression of the PCTA-1 gene, with a modified or forthcoming production of the PCTA-1 protein, or with the production of a modified PCTA-1 protein.

In a preferred embodiment, the invention relates to a method for the screening of candidate substances for cancer treatment, preferably prostate cancer treatment. The method comprises the following steps:

- providing a cell line, an organ, or a mammal expressing a *PCTA-1* gene or a fragment thereof, preferably the regulatory region or the promoter region of the *PCTA-1* gene;
- obtaining a candidate substance, preferably a candidate substance capable of inhibiting the binding of a transcription factor to the *PCTA-1* regulatory region; and
- testing the ability of the candidate substance to decrease the symptoms of cancer, preferably of prostate cancer and/or to modulate the expression levels of *PCTA-1*.

In some embodiments, the cell line, organ or mammal expresses a heterologous protein, the coding sequence of which is operably linked to the *PCTA-1* regulatory or promoter sequence. In other embodiments, they express a *PCTA-1* gene comprising alleles of one or more biallelic markers associated with cancer, preferably with prostate cancer, an early onset of prostate cancer, or the aggressiveness of prostate cancer tumors, or a mutated *PCTA-1* gene comprising a trait causing mutation determined using the above-noted method. Optionally, the

biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof. Preferably, the biallelic marker is selected from the group consisting of A2, A30, A41, A55, A57 and the complements thereof. In a further embodiment, a mice expressing a PCTA-1 protein, preferably a mouse PCTA-1 protein encoded by a nucleic acid sequence of SEQ ID No 9 or a variant or a fragment thereof can be used to screen agents acting against cancer, preferably prostate cancer.

A candidate substance is a substance which can interact with or modulate, by binding or other intermolecular interactions, expression, stability, and function of *PCTA-1*. Such substances may be potentially interesting for patients who are not responsive to existing drugs or develop side effects to them. Screening may be effected using either *in vitro* methods or *in vivo* methods.

Such methods can be carried out in numerous ways such as on transformed cells which express the considered alleles of the *PCTA-1* gene, on tumors induced by said transformed cells, for example in mice, or on PCTA-1 protein encoded by the considered allelic variant of *PCTA-1*. This method preferably includes preparing transformed cells with different forms of *PCTA-1* sequences containing particular alleles of one or more biallelic markers and/or trait causing mutations described above. Optionally, the biallelic marker is selected from the group consisting of A1 to A125 and the complements thereof.

Screening assays of the present invention generally involve determining the ability of a candidate substance to present a cytotoxic effect, to change the characteristics of transformed cells such as proliferative and invasive capacity, to affect the tumor growth, or to modify the expression level of *PTCA-1*.

Typical examples of such drug screening assays are provided below. It is to be understood that the parameters set forth in these examples can be modified by the skilled person without undue experimentation.

Screening Substances Interacting With The Regulatory Sequences Of The *PCTA-1* Gene.

The present invention also concerns a method for screening substances or molecules that are able to interact with the regulatory sequences of the *PCTA-1* gene, such as for example promoter or enhancer sequences.

Nucleic acids encoding proteins which are able to interact with the regulatory sequences of the *PCTA-1* gene, more particularly a nucleotide sequence selected from the group consisting of the polynucleotides of the 5' and 3' regulatory region or a fragment or variant thereof, and preferably a variant comprising one of the biallelic markers of the invention, may be identified by using a one-hybrid system, such as that described in the booklet enclosed in the Matchmaker

One-Hybrid System kit from Clontech (Catalog Ref. n° K1603-1). Briefly, the target nucleotide sequence is cloned upstream of a selectable reporter sequence and the resulting DNA construct is integrated in the yeast genome (*Saccharomyces cerevisiae*). The yeast cells containing the reporter sequence in their genome are then transformed with a library consisting of fusion molecules between cDNAs encoding candidate proteins for binding onto the regulatory sequences of the *PCTA-1* gene and sequences encoding the activator domain of a yeast transcription factor such as GAL4. The recombinant yeast cells are plated in a culture broth for selecting cells expressing the reporter sequence. The recombinant yeast cells thus selected contain a fusion protein that is able to bind onto the target regulatory sequence of the *PCTA-1* gene. Then, the cDNAs encoding the fusion proteins are sequenced and may be cloned into expression or transcription vectors *in vitro*. The binding of the encoded polypeptides to the target regulatory sequences of the *PCTA-1* gene may be confirmed by techniques familiar to the one skilled in the art, such as gel retardation assays or DNAse protection assays. Such assays are detailed in the section "Analysis Of Biallelic Markers Of The Invention With Prostate Cancer".

Screening For Expression Modifiers

The *PCTA-1* gene appears to be involved in a series of events which most likely include a modification of at least one step of its transcription process. In fact, and as mentioned previously, there is a strong possibility that this modification is directly related to the binding efficiency of DNA binding factors to sites of the *PCTA-1* regulatory region.

Screening programs can be used to test potentially therapeutic compounds, either by competitively binding to the sites of the *PCTA-1* promoter which would normally bind the DNA transcription factor, or directly binding to the DNA binding factor itself. These compounds could reduce the speed at which the cascade of events leading to the development of *PCTA-1* related cancers takes place. In fact, even though it seems clear that a combination of several DNA binding sites may be involved in the development of a PCTA-1 related prostate cancer, binding inhibition of only a few such sites is likely to be sufficient to significantly impact on PCTA-1 production and hence the proliferation of cancer.

The screening of expression modifiers is important as it can be used for detecting modifiers specific to one allele or a group of alleles of the *PCTA-1* gene. The alteration of *PCTA-1* expression in response to a modifier can be determined by administering or combining the candidate modifier with an expression system such as animals, cells, and *in vitro* transcription assay.

The term "expression modifier" is intended to encompass but is not limited to chemical agents and polypeptides that modulate the action of PCTA-1 through modulation of the PCTA-1 gene expression.

The effect of the modifier on *PCTA-1* transcription and /or steady state mRNA levels can be also determined. As with the basic expression levels, tissue specific interactions are of interest. Correlations are made between the ability of an expression modifier to affect PCTA-1 activity, and the presence of the targeted polymorphisms. A panel of different modifiers may be screened in order to determine the effect under a number of different conditions.

Another subject of the present invention is a method for screening molecules that modulate the expression of the PCTA-1 protein. Such a screening method comprises the steps of:

a) cultivating a prokaryotic or an eukaryotic cell that has been transfected with a nucleotide sequence encoding the PCTA-1 protein or a variant or a fragment thereof, placed under the control of its own promoter;

b) bringing into contact the cultivated cell with a molecule to be tested; and

c) quantifying the expression of the PCTA-1 protein or a variant or a fragment thereof.

In an embodiment, the nucleotide sequence encoding the PCTA-1 protein or a variant or a fragment thereof comprises an allele of at least one of the biallelic markers A1 to A125, preferably A2, A30, A41, A55, A57, and the complements thereof.

Using DNA recombination techniques well known by the one skilled in the art, the PCTA-1 protein encoding DNA sequence is inserted into an expression vector, downstream from its promoter sequence.

The quantification of the expression of the PCTA-1 protein may be realized either at the mRNA level or at the protein level. In the latter case, polyclonal or monoclonal antibodies may be used to quantify the amounts of the PCTA-1 protein that have been produced, for example in an ELISA or a RIA assay.

In a preferred embodiment, the quantification of the *PCTA-1* mRNA is realized by a quantitative PCR amplification of the cDNA obtained by a reverse transcription of the total mRNA of the cultivated *PCTA-1*-transfected host cell, using a pair of primers specific for *PCTA-1*.

Thus, is also part of the present invention a method for screening of a candidate substance or molecule that modulated the expression of the *PCTA-1* gene, this method comprises the following steps:

- providing a recombinant cell host containing a nucleic acid, wherein said nucleic acid comprises a nucleotide sequence of the 5' regulatory region or a biologically active fragment or variant thereof located upstream a polynucleotide encoding a detectable protein;

- obtaining a candidate substance; and

- determining the ability of the candidate substance to modulate the expression levels of the polynucleotide encoding the detectable protein.

In a further embodiment, the nucleic acid comprising the nucleotide sequence of the 5' regulatory region or a biologically active fragment or variant thereof also includes a 5'UTR region of the *PCTA-1* cDNAs, or one of its biologically active fragments or variants thereof.

Among the preferred polynucleotides encoding a detectable protein, there may be cited polynucleotides encoding luciferase, beta galactosidase, green fluorescent protein (GFP) and chloramphenicol acetyl transferase (CAT).

In another embodiment of a method for the screening of a candidate substance or molecule that modulates the expression of the *PCTA-1* gene, wherein said method comprises the following steps:

a) providing a recombinant host cell containing a nucleic acid, wherein said nucleic acid comprises the 5'UTR sequence of a *PCTA-1* cDNA, or one of its biologically active fragments or variants, the 5'UTR sequence or its biologically active fragment or variant being operably linked to a polynucleotide encoding a detectable protein;

b) obtaining a candidate substance; and

c) determining the ability of the candidate substance to modulate the expression levels of the polynucleotide encoding the detectable protein.

In one particular embodiment of the above screening method, the nucleic acid that comprises a nucleotide sequence selected from the group consisting of the 5'UTR sequence of a *PCTA-1* cDNA or one of its biologically active fragments or variants, includes a promoter sequence which is exogenous with respect to the *PCTA-1* 5'UTR sequence defined therein. In a further preferred embodiment, the nucleic acid comprising the 5'-UTR sequence of a *PCTA-1* cDNA or the biologically active fragments thereof includes a biallelic marker selected from the group consisting of A1 to A125, preferably A2, A30, A41, A55, A57, or the complements thereof.

The invention also pertains to kits useful for performing the herein described screening method. Preferably, such kits comprise a recombinant vector that allows the expression of a nucleotide sequence of the 5' regulatory region or a biologically active fragment or variant thereof located upstream and operably linked to a polynucleotide encoding a detectable protein or a PCTA-1 protein or a fragment or a variant thereof. Moreover, the kit can comprise a

recombinant vector that comprises a nucleic acid including a 5'UTR sequence of a *PCTA-1* cDNA, or one of their biologically active fragments or variants, said nucleic acid being operably linked to a polynucleotide encoding a detectable protein or a PCTA-1 protein or a fragment or a variant thereof.

For the design of suitable recombinant vectors useful for performing the screening methods described above, it will be referred to the section of the present specification wherein the preferred recombinant vectors of the invention are detailed.

Expression levels and patterns of *PCTA-1* may be analyzed by solution hybridization with long probes as described in International Patent Application No. WO 97/05277. Briefly, a *PCTA-1* cDNA or the *PCTA-1* genomic DNA described above, or fragments thereof, is inserted at a cloning site immediately downstream of a bacteriophage (T3, T7 or SP6) RNA polymerase promoter to produce antisense RNA. Preferably, the *PCTA-1* insert comprises at least 100 or more consecutive nucleotides of the genomic DNA sequence or a cDNA sequence, particularly those comprising at least one of biallelic markers according the present invention, preferably at least one of the biallelic markers A1 to A125 and the complements thereof or those comprising the trait causing mutation. The plasmid is linearized and transcribed in the presence of ribonucleotides comprising modified ribonucleotides (i.e. biotin-UTP and DIG-UTP). An excess of this doubly labeled RNA is hybridized in solution with mRNA isolated from cells or tissues of interest. The hybridizations are performed under standard stringent conditions (40-50°C for 16 hours in an 80% formamide, 0.4 M NaCl buffer, pH 7-8). The unhybridized probe is removed by digestion with ribonucleases specific for single-stranded RNA (i.e. RNases CL3, T1, Phy M, U2 or A). The presence of the biotin-UTP modification enables capture of the hybrid on a microtitration plate coated with streptavidin. The presence of the DIG modification enables the hybrid to be detected and quantified by ELISA using an anti-DIG antibody coupled to alkaline phosphatase.

Quantitative analysis of the *PCTA-1* gene expression may also be performed using arrays. As used herein, the term array means a one dimensional, two dimensional, or multidimensional arrangement of a plurality of nucleic acids of sufficient length to permit specific detection of expression of mRNAs capable of hybridizing thereto. For example, the arrays may contain a plurality of nucleic acids derived from genes whose expression levels are to be assessed. The arrays may include the *PCTA-1* genomic DNA, a *PCTA-1* cDNA sequence or the sequences complementary thereto or fragments thereof, particularly those comprising at least one of the biallelic markers according the present invention, preferably at least one of the biallelic markers A1 to A125 and the complements thereof or those comprising a trait causing mutation. Preferably, the fragments are at least 15 nucleotides in length. In other embodiments,

the fragments are at least 25 nucleotides in length. In some embodiments, the fragments are at least 50 nucleotides in length. More preferably, the fragments are at least 100 nucleotides in length. In another preferred embodiment, the fragments are more than 100 nucleotides in length. In some embodiments the fragments may be more than 500 nucleotides in length.

For example, quantitative analysis of *PCTA-1* gene expression may be performed with a complementary DNA microarray as described by Schena et al. (1995 and 1996). Full length *PCTA-1* cDNAs or fragments thereof are amplified by PCR and arrayed from a 96-well microtiter plate onto silylated microscope slides using high-speed robotics. Printed arrays are incubated in a humid chamber to allow rehydration of the array elements and rinsed, once in 0.2% SDS for 1 min, twice in water for 1 min and once for 5 min in sodium borohydride solution. The arrays are submerged in water for 2 min at 95°C, transferred into 0.2% SDS for 1 min, rinsed twice with water, air dried and stored in the dark at 25°C.

Cell or tissue mRNA is isolated or commercially obtained and probes are prepared by a single round of reverse transcription. Probes are hybridized to 1 cm^2 microarrays under a 14 x 14 mm glass coverslip for 6-12 hours at 60°C. Arrays are washed for 5 min at 25°C in low stringency wash buffer (1 x SSC/0.2% SDS), then for 10 min at room temperature in high stringency wash buffer (0.1 x SSC/0.2% SDS). Arrays are scanned in 0.1 x SSC using a fluorescence laser scanning device fitted with a custom filter set. Accurate differential expression measurements are obtained by taking the average of the ratios of two independent hybridizations.

Quantitative analysis of PCTA-1 gene expression may also be performed with full length *PCTA-1* cDNAs or fragments thereof in complementary DNA arrays as described by Pietu et al. (1996). The full length *PCTA-1* cDNA or fragments thereof is PCR amplified and spotted on membranes. Then, mRNAs originating from various tissues or cells are labeled with radioactive nucleotides. After hybridization and washing in controlled conditions, the hybridized mRNAs are detected by phospho-imaging or autoradiography. Duplicate experiments are performed and a quantitative analysis of differentially expressed mRNAs is then performed.

Alternatively, expression analysis using the *PCTA-1* genomic DNA, a *PCTA-1* cDNA, or fragments thereof can be done through high density nucleotide arrays as described by Lockhart et al. (1996) and Sosnowsky et al. (1997). Oligonucleotides of 15-50 nucleotides from the sequence of the *PCTA-1* genomic DNA, a *PCTA-1* cDNA sequence, particularly a sequence comprising at least one of biallelic markers according the present invention, preferably at least one of the biallelic markers A1 to A125 and the complements thereof or comprising the trait causing mutation, or a sequence complementary thereto, are synthesized directly on the chip

(Lockhart et al., supra) or synthesized and then addressed to the chip (Sosnowski et al., supra). Preferably, the oligonucleotides are about 20 nucleotides in length.

PCTA-1 cDNA probes labeled with an appropriate compound, such as biotin, digoxigenin or fluorescent dye, are synthesized from the appropriate mRNA population and then randomly fragmented to an average size of 50 to 100 nucleotides. The said probes are then hybridized to the chip. After washing as described in Lockhart et al., supra and application of different electric fields (Sosnowsky et al., 1997)., the dyes or labeling compounds are detected and quantified. Duplicate hybridizations are performed. Comparative analysis of the intensity of the signal originating from cDNA probes on the same target oligonucleotide in different cDNA samples indicates a differential expression of *PCTA-1* mRNA.

Screening For Molecules Interacting With A PCTA-1 Protein

The PCTA-1 proteins or fragments thereof described above may be used in drug screening procedures to identify molecules which are agonists, antagonists, or inhibitors of PCTA-1 activity. In a preferred embodiment, the PCTA-1 proteins or fragments thereof comprise at least one mutation provided either by biallelic markers of the present invention, preferably at least one mutation encoding by the biallelic markers A54, A56, A60, A75, A76, A85, or by a trait causing mutation according to the present invention. The PCTA-1 proteins or fragments thereof used in such analyses may be free in solution or linked to a solid support. Alternatively, the PCTA-1 proteins or fragments thereof can be expressed on a cell surface. The cell may naturally express a PCTA-1 protein or a fragment thereof or, alternatively, the cell may express a PCTA-1 protein or a fragment thereof from an expression vector such as those described above.

In one method of drug screening, eucaryotic or procaryotic host cells which are stably transformed with recombinant polynucleotides in order to express a PCTA-1 protein or a fragment thereof are used in conventional competitive binding assays or standard direct binding assays.

To study the interaction of a PCTA-1 protein or a fragment thereof with drugs or small molecules, such as molecules generated through combinatorial chemistry approaches, the microdialysis coupled to HPLC method described by Wang et al. (1997) or the affinity capillary electrophoresis method described by Bush et al. (1997) can be used.

In further methods, molecules which interact with a PCTA-1 protein or a fragment thereof may be identified using assays such as the following. The molecule to be tested for binding is labeled with a detectable label, such as a fluorescent , radioactive, or enzymatic tag and placed in contact with an immobilized PCTA-1 protein or a fragment thereof under

conditions which permit specific binding to occur. After removal of non-specifically bound molecules, bound molecules are detected using appropriate means.

Another object of the present invention consists of methods and kits for the screening of candidate substances that interact with a PCTA-1 polypeptide.

A method for the screening of a candidate substance comprises the following steps : a) providing a polypeptide consisting of a PCTA-1 protein or a fragment thereof; b) obtaining a candidate substance; c) bringing into contact said polypeptide with said candidate substance; and d) detecting the complexes formed between said polypeptide and said candidate substance. Optionally, said PCTA-1 protein or fragment thereof is selected from the group consisting of polypeptides of SEQ ID Nos 5, 6, 7, 9 and fragments thereof.

The invention also pertains to kits useful for performing the hereinbefore described screening method. Preferably, such kits comprise a PCTA-1 polypeptide or a fragment thereof, and optionally means useful to detect the complex formed between a PCTA-1 polypeptide or a fragment thereof and the candidate substance. In a preferred embodiment the detection means consist in monoclonal or polyclonal antibodies directed against the corresponding PCTA-1 polypeptide or a fragment thereof.

Various candidate substances or molecules can be assayed for interaction with a PCTA-1 protein or a fragment thereof. These substances or molecules include, without being limited to, natural or synthetic organic compounds or molecules of biological origin such as polypeptides, antibodies, fatty acids and lipoproteins. When the candidate substance or molecule consists of a polypeptide, this polypeptide may be the resulting expression product of a phage clone belonging to a phage-based random peptide library, or alternatively the polypeptide may be the resulting expression product of a cDNA library cloned in a vector suitable for performing a two-hybrid screening assay.

For the purpose of the present invention, a ligand means a molecule, such as a protein, a peptide, an antibody, a fatty acid, a lipoprotein, or any synthetic chemical compound capable of binding to a PCTA-1 protein or a fragment thereof.

A. Candidate Ligands Obtained From Random Peptide Libraries

In a particular embodiment of the screening method, the putative ligand is the expression product of a DNA insert contained in a phage vector (Parmley and Smith, 1988). Specifically, random peptide phages libraries are used. The random DNA inserts encode for peptides of 8 to 20 amino acids in length (Oldenburg K.R. et al., 1992; Valadon P., et al., 1996; Lucas A.H., 1994; Westerink M.A.J., 1995; Felici F. et al., 1991). According to this particular embodiment, the recombinant phages expressing a protein that binds to the immobilized PCTA-

1 protein or a fragment thereof is retained and the complex formed between the PCTA-1 polypeptide and the recombinant phage may be subsequently immunoprecipitated by a polyclonal or a monoclonal antibody directed against the PCTA-1 polypeptide.

Once the ligand library in recombinant phages has been constructed, the phage population is brought into contact with the immobilized PCTA-1 protein or a fragment thereof. Then the preparation of complexes is washed in order to remove the non-specifically bound recombinant phages. The phages that bind specifically to the PCTA-1 protein or a fragment thereof are then eluted by a buffer (acid pH) or immunoprecipitated by the monoclonal antibody produced by the hybridoma anti-PCTA-1, and this phage population is subsequently amplified by an over-infection of bacteria (for example E. coli). The selection step may be repeated several times, preferably 2-4 times, in order to select the more specific recombinant phage clones. The last step consists of characterizing the peptide produced by the selected recombinant phage clones either by expression in infected bacteria and isolation, expressing the phage insert in another host-vector system, or sequencing the insert contained in the selected recombinant phages.

B. Candidate Ligands Obtained By Competition Experiments.

Alternatively, peptides, drugs or small molecules which bind to the PCTA-1 protein, or a fragment thereof may be identified in competition experiments. In such assays, the PCTA-1 protein or a fragment thereof is immobilized to a surface, such as a plastic plate. Increasing amounts of the peptides, drugs or small molecules are placed in contact with the immobilized PCTA-1 protein or a fragment thereof in the presence of a detectable labeled known PCTA-1 protein ligand. For example, the PCTA-1 ligand may be detectably labeled with a fluorescent, radioactive, or enzymatic tag. The ability of the test molecule to bind the PCTA-1 protein or a fragment thereof is determined by measuring the amount of detectably labeled known ligand bound in the presence of the test molecule. A decrease in the amount of known ligand bound to the PCTA-1 protein or a fragment thereof when the test molecule is present indicated that the test molecule is able to bind to the PCTA-1 protein or a fragment thereof.

C. Candidate Ligands Obtained By Affinity Chromatography.

Proteins or other molecules interacting with the PCTA-1 protein or a fragment thereof can also be found using affinity columns which contain the PCTA-1 protein or a fragment thereof. The PCTA-1 protein or a fragment thereof may be attached to the column using conventional techniques including chemical coupling to a suitable column matrix such as agarose, Affi Gel® , or other matrices familiar to those of skill in art. In some embodiments of this method, the affinity column contains chimeric proteins in which the PCTA-1 protein or a

fragment thereof is fused to glutathion S transferase (GST). A mixture of cellular proteins or pool of expressed proteins as described above is applied to the affinity column. Proteins or other molecules interacting with the PCTA-1 protein or a fragment thereof attached to the column can then be isolated and analyzed on 2-D electrophoresis gel as described in Ramunsen et al. (1997). Alternatively, the proteins retained on the affinity column can be purified by electrophoresis based methods and sequenced. The same method can be used to isolate antibodies, to screen phage display products, or to screen phage display human antibodies.

D. Candidate Ligands Obtained By Optical Biosensor methods

Proteins interacting with the PCTA-1 protein or a fragment thereof can also be screened by using an Optical Biosensor as described in Edwards and Leatherbarrow (1997) and also in Szabo et al. (1995). This technique permits the detection of interactions between molecules in real time, without the need of labeled molecules. This technique is based on the surface plasmon resonance (SPR) phenomenon. Briefly, the candidate ligand molecule to be tested is attached to a surface (such as a carboxymethyl dextran matrix). A light beam is directed towards the side of the surface that does not contain the sample to be tested and is reflected by said surface. The SPR phenomenon causes a decrease in the intensity of the reflected light with a specific association of angle and wavelength. The binding of candidate ligand molecules cause a change in the refraction index on the surface, which change is detected as a change in the SPR signal. For screening of candidate ligand molecules or substances that are able to interact with the PCTA-1 protein or a fragment thereof, the PCTA-1 polypeptide is immobilized onto a surface. This surface consists of one side of a cell through which flows the candidate molecule to be assayed. The binding of the candidate molecule on the PCTA-1 protein or a fragment thereof is detected as a change of the SPR signal. The candidate molecules tested may be proteins, peptides, carbohydrates, lipids, or small molecules generated by combinatorial chemistry. This technique may also be performed by immobilizing eukaryotic or prokaryotic cells or lipid vesicles exhibiting an endogenous or a recombinantly expressed PCTA-1 protein at their surface.

The main advantage of the method is that it allows the determination of the association rate between the PCTA-1 protein and molecules interacting with the PCTA-1 protein. It is thus possible to select specifically ligand molecules interacting with the PCTA-1 protein, or a fragment thereof, through strong or conversely weak association constants.

E. Candidate Ligands Obtained Through A Two-Hybrid Screening Assay.

The yeast two-hybrid system is designed to study protein-protein interactions *in vivo* (Fields and Song, 1989), and relies upon the fusion of a bait protein to the DNA binding domain

of the yeast Gal4 protein. This technique is also described in the US Patent N° US 5,667,973 and the US Patent N° 5,283,173 (Fields et al.), the disclosures of which are incorporated herein by reference in their entireties.

The general procedure of library screening by the two-hybrid assay may be performed as described by Harper et al. (1993) or as described by Cho et al. (1998) or also Fromont-Racine et al. (1997).

The bait protein or polypeptide consists of a PCTA-1 polypeptide or a fragment thereof.

More precisely, the nucleotide sequence encoding the PCTA-1 polypeptide or a fragment thereof is fused to a polynucleotide encoding the DNA binding domain of the GAL4 protein, the fused nucleotide sequence being inserted in a suitable expression vector, for example pAS2 or pM3.

Then, a human cDNA library is constructed in a specially designed vector, such that the human cDNA insert is fused to a nucleotide sequence in the vector that encodes the transcriptional domain of the GAL4 protein. Preferably, the vector used is the pACT vector. The polypeptides encoded by the nucleotide inserts of the human cDNA library are termed "pray" polypeptides.

A third vector contains a detectable marker gene, such as beta galactosidase gene or CAT gene that is placed under the control of a regulation sequence that is responsive to the binding of a complete Gal4 protein containing both the transcriptional activation domain and the DNA binding domain. For example, the vector pG5EC may be used.

Two different yeast strains are also used. As an illustrative but non limiting example the two different yeast strains may be the followings :

Y190, the phenotype of which is (*MATa, Leu2-3, 112 ura3-12, trp1-901, his3-D200, ade2-101, gal4Dgal180D URA3 GAL-LacZ, LYS GAL-HIS3, cyh*^{r});

Y187, the phenotype of which is (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 URA3 GAL-lacZmet*$^-$), which is the opposite mating type of Y190.

Briefly, 20 µg of pAS2/*PCTA-1* and 20 µg of pACT-cDNA library are co-transformed into yeast strain Y190. The transformants are selected for growth on minimal media lacking histidine, leucine and tryptophan, but containing the histidine synthesis inhibitor 3-AT (50 mM). Positive colonies are screened for beta galactosidase by filter lift assay. The double positive colonies (*His*$^+$, *beta-gal*$^+$) are then grown on plates lacking histidine, leucine, but containing tryptophan and cycloheximide (10 mg/ml) to select for loss of pAS2/*PCTA-1* plasmids bu retention of pACT-cDNA library plasmids. The resulting Y190 strains are mated with Y187 strains expressing PCTA-1 or non-related control proteins; such as cyclophilin B, lamin, or SNF1, as *Gal4* fusions as described by Harper et al. (1993) and by Bram et al. (1993),

and screened for beta galactosidase by filter lift assay. Yeast clones that are *beta gal-* after mating with the control *Gal4* fusions are considered false positives.

In another embodiment of the two-hybrid method according to the invention, interaction between the PCTA-1 or a fragment thereof with cellular proteins may be assessed using the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech). As described in the manual accompanying the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech), nucleic acids encoding the PCTA-1 protein or a fragment thereof, are inserted into an expression vector such that they are in frame with DNA encoding the DNA binding domain of the yeast transcriptional activator GAL4. A desired cDNA, preferably human cDNA, is inserted into a second expression vector such that they are in frame with DNA encoding the activation domain of GAL4. The two expression plasmids are transformed into yeast and the yeast are plated on selection medium which selects for expression of selectable markers on each of the expression vectors as well as GAL4 dependent expression of the HIS3 gene. Transformants capable of growing on medium lacking histidine are screened for GAL4 dependent lacZ expression. Those cells which are positive in both the histidine selection and the lacZ assay contain interaction between PCTA-1 and the protein or peptide encoded by the initially selected cDNA insert.

Screening Through Spontaneous Metastatic Assay

Screening of new compounds can be carried out through a spontaneous metastatic assay as described in Nihei et al. (1995). Hence, it can be possible to assess the decrease of metastatic potential of transformed cells related to treatment of said compounds. Indeed, according to the present invention, the metastatic potential of cells constitutes the major criteria of the aggressiveness of prostate cancer tumors.

To evaluate the metastatic ability, about $5x10^5$ cells expressing a *PCTA-1* gene comprising alleles for one or more biallelic markers associated with cancer, preferably with prostate cancer, or with the aggressiveness of prostate cancer tumors, are injected subcutaneously in the flank of male athymic nude mice. The mice are treated with the screened compounds. Tumor volume and tumor volume doubling time are used as the index of the tumor growth rate and are determined as described in Isaacs & Hukku, 1988). The tumor-bearing animals are scored for lung metastases at spontaneous death or when killed at day 35 post-inoculation.

Gene Therapy

Gene therapy involves the alteration of the phenotypic expression of a targeted cell, usually a cancer cell through the alteration of the cell's genotypic content. The desired effect of gene therapy is a reduction or interruption of tumor growth or, ideally, the destruction of the cell

itself. An appropriate gene for gene therapy must be capable of altering the biological behavior of the cancer cell in order to slow growth, reduce local invasive potential, or induce apoptosis. The *PCTA-1* gene, or certain portions thereof, is a good candidate for gene therapy.

The present invention also comprises the use of the genomic *PCTA-1* DNA described above or a fragment thereof, in gene therapy strategies, such as antisense and triple helix strategies, and in the introduction of a therapeutic gene. Preferred nucleotide sequences useful in gene therapy include the sequences of SEQ ID Nos 1, 2, 3, 4, 8, complementary sequences thereto, and fragments thereof. More particularly, preferred nucleotide sequences comprise any of the polynucleotides described in the "*PCTA-1* cDNA Sequences" section, the "Coding Regions" section, and the "Oligonucleotide Probes And Primers" section. Preferred *PCTA-1* DNA fragments used in such approaches are those comprising a nucleotide sequence comprising a *PCTA-1* regulatory region or a fragment thereof. More particularly, the regulatory regions comprise at least one of the biallelic markers according to the invention, more particularly those comprising a biallelic marker selected from the group consisting of A1 to A125, preferably A2, A30, A41, A55, A57, or a trait causing mutation, or complementary sequences thereof or variants or fragments thereof.

In a first embodiment, the invention therefore concerns a method for the treatment of prostate cancer. The method comprises: (a) selecting an individual whose DNA comprises an allele of biallelic marker or of a group of biallelic markers, preferably markers of the *PCTA-1* gene, associated with a susceptibility to prostate cancer; and (b) administering to the individual an effective amount of a molecule capable of modifying the expression of the *PCTA-1* gene.

In one embodiment, the molecule is an antisense nucleotide sequence, capable of competitively binding to the mRNA sequence resulting from the transcription of the *PCTA-1* genomic DNA so as to prevent the translation of said mRNA. In preferred embodiments of this method, the antisense nucleotide sequence is characterized in that it hybridizes with exons of the *PCTA-1* gene, preferably with a region of such exons comprising a least an allele of one of the biallelic markers of the present invention. Optionally, the antisense nucleotide sequence hybridizes with exons 0, 1, 6bis, 9 or 9ter of the *PCTA-1* gene.

In an other embodiment, the molecule is a nucleotide sequence comprising a homopurine or homopyridine, preferably a 10-mer to 20-mer homopurine or homopyridine, which is complementary to a homopurine or homopyridine sequence of the *PCTA-1* genomic DNA so as to prevent transcription of said genomic DNA into mRNA.

In a further embodiment, the molecule is a nucleotide sequence comprising a DNA sequence encoding a protein capable, when expressed, of exerting a therapeutic effect on

prostate cancer cells, operably linked to the promoter of *PCTA-1* gene, so as to kill or disable said prostate cancer cells.

The invention also concerns a method for the treatment of prostate cancer comprising:

- administering to an individual an effective amount of a nucleotide sequence comprising a DNA sequence encoding a protein capable, when expressed, of exerting a therapeutic effect on prostate cancer cells, operably linked to the promoter of *PCTA-1* gene.

The gene encoding a protein capable of exerting a therapeutic effect on prostate cancer cells is called the therapeutic gene in the present application. In some embodiments, the therapeutic gene is a toxin gene encoding a cytotoxic or cytostatic gene product. In another embodiment, the therapeutic gene is a gene encoding an immunogenic antigen which is highly visible to the immune system. In further embodiment, the therapeutic gene is a gene encoding a lymphokine which activates an anti-tumor immune response. In additional embodiments, the therapeutic gene encodes an antisense sequence having as a target the coding region of an essential gene for the proliferation or viability of the cell.

Antisense Approach

In antisense approaches, nucleic acid sequences complementary to a targeted mRNA are hybridized to the mRNA intracellularly, thereby blocking the expression of the protein encoded by the mRNA. The antisense sequences can prevent gene expression through a variety of mechanisms. For example, the antisense sequences may inhibit the ability of ribosomes to translate the mRNA. Alternatively, the antisense sequences may block transport of the mRNA from the nucleus to the cytoplasm, thereby limiting the amount of mRNA available for translation. Another mechanism through which antisense sequences may inhibit gene expression is by interfering with mRNA splicing. In yet another strategy, the antisense nucleic acid may be incorporated in a ribozyme capable of specifically cleaving the target mRNA.

The antisense nucleic acid molecules to be used in gene therapy may be either DNA or RNA sequences. They comprise a nucleotide sequence complementary to the targeted sequence of the *PCTA-1* genomic DNA or a *PCTA-1* cDNA. The targeted DNA or RNA sequence preferably comprises at least one of the biallelic markers according to the present invention, particularly a biallelic marker selected from the group consisting of A1 to A125 and the complements thereof, or comprises a trait causing mutation. In a preferred embodiment, the antisense oligonucleotide are able to hybridize with at least one of the splicing sites of the targeted PCTA-1 gene, with the 3'UTR or the 5'UTR, with exon 0, 1, 6bis, 9 or 9ter, or with an exonic region comprising at least one of the biallelic markers of the present invention or comprising a trait causing mutation.

Preferred methods using antisense polynucleotide according to the present invention are the procedures described by Sczakiel et al.(1995).

Preferably, the antisense tools are chosen among the polynucleotides (15-200 bp long) that are complementary to the 5'end of the *PCTA-1* mRNA. In another embodiment, a combination of different antisense polynucleotides complementary to different parts of the desired targeted gene are used.

Preferred antisense polynucleotides according to the present invention are complementary to a sequence of the mRNAs of *PCTA-1* that contains either the translation initiation codon ATG or a splicing donor or acceptor site.

The antisense nucleic acids should have a length and melting temperature sufficient to permit formation of an intracellular duplex having sufficient stability to inhibit the expression of the *PCTA-1* mRNA in the duplex. Strategies for designing antisense nucleic acids suitable for use in gene therapy are disclosed in Green et al., (1986) and Izant and Weintraub, (1984).

In some strategies, antisense molecules are obtained by reversing the orientation of the *PCTA-1* coding region with respect to a promoter so as to transcribe the opposite strand from that which is normally transcribed in the cell. The antisense molecules may be transcribed using in vitro transcription systems such as those which employ T7 or SP6 polymerase to generate the transcript. Another approach involves transcription of *PCTA-1* antisense nucleic acids in vivo by operably linking DNA containing the antisense sequence to a promoter in a suitable expression vector.

Alternatively, suitable antisense strategies are those described by Rossi et al.(1991), in the International Applications Nos. WO 94/23026, WO 95/04141, WO 92/18522, WO 96/31523 and in the European Patent Application No. EP 0 572 287 A2, the disclosures of which are incorporated herein by reference in their entireties.

An alternative to the antisense technology that is used according to the present invention consists of using ribozymes that will bind to a target sequence via their complementary polynucleotide tail and that will cleave the corresponding RNA by hydrolyzing its target site (namely "hammerhead ribozymes"). Briefly, the simplified cycle of a hammerhead ribozyme consists of (1) sequence specific binding to the target RNA via complementary antisense sequences; (2) site-specific hydrolysis of the cleavable motif of the target strand; and (3) release of cleavage products, which gives rise to another catalytic cycle. Indeed, the use of long-chain antisense polynucleotide (at least 30 bases long) or ribozymes with long antisense arms are advantageous. A preferred delivery system for antisense ribozyme is achieved by covalently linking these antisense ribozymes to lipophilic groups or to use liposomes as a convenient

vector. Preferred antisense ribozymes according to the present invention are prepared as described by Sczakiel et al.(1995).

Triple Helix Approach

The *PCTA-1* genomic DNA, preferably comprising at least one of the biallelic markers according to the invention, more preferably at least one biallelic marker selected from the group consisting of A1 to A125, or comprising a trait causing mutation, or complementary sequences, variants or fragments thereof, may also be used in gene therapy approaches based on intracellular triple helix formation.

Triple helix oligonucleotides are used to inhibit transcription from a genome. They are particularly useful for studying alterations in cell activity when it is associated with a particular gene. Fragments of the *PCTA-1* genomic DNA can be used to inhibit gene expression in individuals suffering from prostate cancer or from another detectable phenotype, or in individuals at risk of developing prostate cancer or another detectable phenotype at a later date as a result of their *PCTA-1* genotype.

Similarly, a portion of the *PCTA-1* genomic DNA can be used to study the effect of inhibiting PCTA-1 transcription within a cell. Traditionally, homopurine sequences were considered the most useful for triple helix strategies. However, homopyrimidine sequences can also inhibit gene expression. Such homopyrimidine oligonucleotides bind to the major groove at homopurine:homopyrimidine sequences. Thus, both types of sequences from the *PCTA-1* genomic DNA, preferably comprising at least one of the biallelic markers according to the invention, more preferably at least one of the biallelic markers A1 to A125, or comprising the trait causing mutation, or complementary sequences thereof, variants thereof, are contemplated within the scope of this invention.

To carry out gene therapy strategies using the triple helix approach, the sequences of the *PCTA-1* genomic DNA, preferably comprising at least one of the biallelic markers according to the invention, or comprising the trait causing mutation, or complementary sequences thereof, or variants thereof, are first scanned to identify 10-mer to 20-mer homopyrimidine or homopurine stretches which could be used in triple-helix based strategies for inhibiting PCTA-1 expression. Following identification of candidate homopyrimidine or homopurine stretches, their efficiency in inhibiting PCTA-1 expression is assessed by introducing varying amounts of oligonucleotides containing the candidate sequences into tissue culture cells which express the PCTA-1 gene.

The oligonucleotides can be introduced into the cells using a variety of methods known to those skilled in the art, including but not limited to calcium phosphate precipitation, DEAE-Dextran, electroporation, liposome-mediated transfection or native uptake.

Treated cells are monitored for altered cell function or reduced PCTA-1 expression using techniques such as Northern blotting, RNase protection assays, or PCR based strategies to monitor the transcription levels of the *PCTA-1* gene in cells which have been treated with the oligonucleotide.

The oligonucleotides which are effective in inhibiting gene expression in tissue culture cells may then be introduced in vivo using the techniques described above in the antisense approach at a dosage calculated based on the in vitro results, as described in antisense approach.

In some embodiments, the natural (beta) anomers of the oligonucleotide units can be replaced with alpha anomers to render the oligonucleotide more resistant to nucleases. Further, an intercalating agent such as ethidium bromide, or the like, can be attached to the 3′ end of the alpha oligonucleotide to stabilize the triple helix. For information on the generation of oligonucleotides suitable for triple helix formation see Griffin et al. (1989).

Introduction Of A Therapeutic Gene

One important aspect of the present invention concerns a promoter specifically expressed in prostate cancer cells. More particularly, the present invention relates to the regulatory sequences, and particularly the promoter of the *PCTA-1* gene. The expression of *PCTA-1* appears to be specific to prostate cancer cells.

The term "specific", when used herein with reference to a promoter, is intended to designate a promoter which is specifically expressed in prostate cancer cells, at a level which is sufficient to have a significant impact on the metabolism of such cells. In other words, the promoter is specific in activity, effect or function. However, the term does not necessarily designate a promoter which is expressed solely in prostate cancer cells. Indeed, it is possible that the *PCTA-1* gene is expressed, under the control of its promoter, in other cells at levels which are sufficiently low to be undetectable by current detection techniques such as those involving antibodies, hybridization with a probe or even PCR. The promoter of the *PCTA-1* gene can be advantageously used to introduce a therapeutic gene which will be expressed specifically in prostate cancer cells.

The invention therefore also concerns an expression vector comprising a DNA sequence encoding a functional protein, particularly a functional protein capable of exerting a therapeutic effect on prostate cancer cells, operably linked to the promoter of the *PCTA-1* gene which is specifically expressed in prostate cancer cells.

Furthermore, the *PCTA-1* promoter preferably comprises biallelic markers according to the invention, more particularly those described previously. Some alleles of the biallelic markers of the invention show an association with prostate cancer and may be involved in a modified or forthcoming expression of the *PCTA-1* gene in prostate cancer cells. It may therefore advantageous to use the PCTA-1 promoter comprising such an allele to introduce a therapeutic gene for enhancing its expression in prostate cancer cells.

The term "therapeutic gene" is intended to designate DNA encoding an amino acid sequence corresponding to a functional peptide or protein capable of exerting a therapeutic effect on prostate cancer cells preferably by killing or disabling such cells, or having a regulatory effect on the expression of an important function in prostate cells.

In one embodiment, a single enhancer element or multiple enhancer elements which amplify the expression of the therapeutic gene without compromising tissue specificity can also be combined with the promoter of the *PCTA-1* gene. In a preferred embodiment, the enhancer element may be a portion of the cytomegalovirus LTR, SV40 enhancer sequences, or MMTV LTR. Preferably, the enhancer element is positioned upstream of the PCTA-1 promoter.

The term "enhancer element" is intended to designate a nucleotide sequence that increases the rate of transcription of therapeutic genes or genes of interest but does not have promoter activity. An enhancer can be moved upstream, downstream, and to the other side of the PCTA-1 promoter without significant loss of activity.

In a preferred embodiment, a vector is constructed by inserting the therapeutic gene downstream of the PCTA-1 promoter. The therapeutic gene is inserted so as to be operably linked to the promoter.

Examples of therapeutic genes include suicide genes. These are gene sequences, the expression of which produces a protein or agent that inhibits prostate tumor cell growth or induces prostate tumor cell death. Genes of interest include genes encoding enzymes, oncogenes, tumor suppressor genes, genes encoding toxins, genes encoding cytokines, or a gene encoding oncostatin. The purpose of the therapeutic genes is to inhibit the growth of or kill prostate cancer cells or to produce cytokines or other cytotoxic agents which directly or indirectly inhibit the growth of or kill prostate cancer cell.

Suitable enzymes include thymidine kinase, xanthine-guanine phosphoribosyltransferase, cytosine deaminase, and hypoxanthine phosphoribosyl transferase. Suitable oncogenes and tumor suppressor genes include neu, EGF, ras, p53, retinoblastoma tumor suppressor gene (Rb), Wilm's tumor gene product, phosphotyrosine phosphatase, and nm23. Suitable toxins include Pseudomonas exotoxin A and S, diphteria toxin, E. coli LT

toxins, Shiga toxin, Shiga-like toxins, ricin, abrin, supporin, and gelonin. Suitable cytokines include interferons, GM-CSF interleukins, tumor necrosis factor.

Other gene therapy strategies include antisense sequences as mentioned above of at least about 30 bp, preferably 50 pb, having as target the coding sequence of an essential gene for the proliferation or viability of the cell. Numerous proteins associated with transcription, translation, metabolic pathways, cytostructural genes can be used as target, preferably those which are essential, present at relatively low levels, and particularly associated with cancer cells.

The three presently available methodologies for DNA delivery are well-known by the skilled artisan: transfection with a viral vector; fusion with a lipid; and cationic supported DNA introduction. A suitable DNA delivery method should meet the following criteria: 1) capable of directing the therapeutic polynucleotides into specific target cell types, 2) highly efficient in mediating uptake of the therapeutic polynucleotide into the target cells, and 3) suited for use in vivo for therapeutic application.

The preferred method relies on replication-defective viral vectors harboring the therapeutic polynucleotide sequence as part of retroviral genome. Preferred vectors for use in the present invention are viral including adenoviruses, retroviral vectors, and adeno-associated viral vectors. Retroviral vectors and adenoviruses offer an efficient, useful, and presently the best-characterized means of introducing and expressing foreign genes efficiently in mammalian cells. These vectors have very broad host and cell type range and express genes stably and efficiently.

Other virus vectors that may be used for gene transfer into cells include retroviruses such as Moloney murine lekemia virus, papovaviruses such as JC, SV40, polyoma, and adenoviruses, Epstein-Barr virus, papilloma viruses such as bovine papilloma virus type I, vaccinia, and poliovirus.

Another gene transfer method is physical transfer of plasmid DNA comprising the therapeutic polynucleotide in liposomes directly into prostate, preferably into tumors cells in situ. Immunoliposomes may improve cell type specificity as compared to liposomes by virtue of the inclusion of specific antibodies which presumably bind to surface antigens specific of prostate cells. In one embodiment, antibodies are directed against PCTA-1 protein which is specific to prostate cancer cells.

Direct physical application of naked DNA comprising the therapeutic polynucleotide to the target cells is believed to be preferred in many cases.

Vaccine composition

The invention concerns a vaccine composition comprising a vaccination agent including one of the following polypeptide:

a) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5 , wherein said contigous span comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5;

b) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6 , wherein said contigous span comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6;

c) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7 , wherein said contigous span comprises:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7; and

d) a polypeptide comprising a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9.

"Vaccine agent or vaccination agent" is intended to designate a substance which has the ability, when administered to a patient in suitable amounts, to generate an immunogenic reaction which can confer either immunity to the patent against prostate cancer or kill or disable prostate cancer cells bearing on their surface the PCTA-1 protein or a fragment thereof.

The vaccine compositions of the present invention are intended to be administered to patients in an amount sufficient to inhibit the growth of cancer cells expressing the PCTA-1 protein. More particularly the vaccine composition is intended to decrease the growth rate, rate of division, or viability of the prostate cancer cells.

The administration of a vaccine composition of the invention may be for either a "prophylactic" or "therapeutic" purposes. When provided prophylactically, the vaccine agent are provided in advance of symptoms indicative of prostate cancer. The prophylactic administration of vaccine agent serves to prevent, attenuate, or inhibit of the growth of prostate cancer cells. The therapeutic administration of the vaccine agent serves to attenuate the pathological symptoms of prostate cancer, to decrease the size or growth of cancer tumors and or metastasis or to remove them.

The term "inhibition of growth" refers in the present application to the decrease of the rate of growth, rate of division, or viability of the cells in question.

Indeed, as the PCTA-1 gene is specifically expressed in prostate cancer cells, these vaccine agents can initiate the production of PCTA-1 specific cytotoxic T lymphocytes which lyse cells bearing, preferably on their surface, PCTA-1, a fragment of PCTA-1, or one or more PCTA-1 epitope peptides thereof and which lead to an inhibition of the growth of cancer also bearing the PCTA-1 protein.

Vaccine preparations which contain protein or peptide sequences as active substances are generally well known in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, the disclosures of which are incorporated herein by reference in their entireties.

A vaccine according to the present invention may further contain auxiliary vaccine constituents, such as carriers, buffers, stabilizers, solubilizers, adjuvants and preservatives.

In order to enhance the immunogenic character of the polypeptides taken from the mutated PCTA-1 protein, the polypeptides may be prepared as homopolymers (a multitude of identical polypeptides coupled to one another) or heteropolymers (a multitude of at least two different polypeptides coupled to one another).

The vaccine agents of the present invention can be used in native form or can be modified to form a chemical derivative. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc... The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, et. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980).

The vaccine agents of the present invention may be administered in a convenient manner such as by oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, or intradermal routes. The vaccine agents of the present invention are administered in an amount which is effective for treatment and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10 μg/kg body weight per day and in most cases they are administered in an amount not in excess of about 8 mg/kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

When administering the vaccine agent of the present invention to a patient, the dosage of the administered vaccine agent varies depending upon such factors as the patient's age, weight, sex, general medical condition, previous medical history. In general, it is desirable to provide the recipient with a dosage of vaccine agent which is in the range of from about 1 pg/kg to 10 mg/kg body weight, although a lower or higher dosage may be administered. The therapeutically effective dose can be lowered by using combinations of the vaccine agents of the present invention or other agents.

It is normally necessary to have multiple administrations of the vaccine agents, usually not exceeding six vaccinations, more usually not exceeding four vaccinations, preferably one or more vaccinations, more preferably about three vaccinations. The vaccinations will be normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years will be desirable to maintain levels of protective immunity.

The vaccine agents of the present invention are intended to be provided to recipient subjects in an amount sufficient to inhibit the growth (as defined above) of cancer cells bearing PCTA-1 protein.

The effect of the vaccine agents of the present invention can be assessed through the measurement of released IFN-γ from memory T-lymphocytes. The stronger of the immune response, the more IFN-γ will be released. Accordingly, a vaccine according to the invention comprises a polypeptide capable of releasing from the memory T-lymphocytes at least 1500 pg/ml, preferably 200 pg/ml, and more preferably 300 pg/ml of IFN-γ. Practically, the levels of IFN-γ from the primed lymphocytes are measured with in vitro proliferation assays of peripheral blood lymphocytes co-cultured with a vaccine agents to be tested. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent 3,791,932; 4,174,384; and 3,949,064, the disclosures of which are incorporated herein by reference in their entireties, as illustrative of these types of assays.

The administration of the vaccine agent of the invention may be for either a "prophylactic" or "therapeutic" purposes. When provided prophylactically, the vaccine agent are administered in advance of any symptoms indicative of prostate cancer. The prophylactic administration of the vaccine agent serves to prevent, attenuate, or inhibit of the growth of prostate cancer cells. The therapeutic administration of the vaccine agent serves to attenuate the pathological symptoms of prostate cancer and to decrease the size of prostate cancer tumors or to remove them.

Typically, such vaccine agents are prepared as injectable either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the vaccine agent. Suitable excipients are, for example, water, saline, dextrose, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

PCTA-1 protein and peptides, preferably mutated, may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts which are formed between the free amino groups of the peptide, and inorganic acids, such as hydrochloric or phosphoric acids, or organic acids, such as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as sodium, potassium, ammonium, calcium, or ferric hydroxydes, or from organic bases such as isopropylamine, trimethylamine, 2-ethylaminoethanol, histidine, procaine, and the like.

Some of the polypeptides of the vaccine agents of the invention are sufficiently immunogenic in a vaccine, but the immune response can be enhanced if the vaccine further comprises an adjuvant substance.

Various methods of achieving adjuvant effects for vaccines include the use of agents such as aluminim hydroxide or phosphate, commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperature ranging between 70°C and 101°C for 30 second to 2 minute periods, respectively. Aggregation by reacting with pepsin treated antibodies (Fab) to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide monoleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. According to the invention, dimethyldioctadecylammonium bromide is an interesting candidate for an adjuvant, but also Freund's complete and incomplete adjuvants as well as QuilA and RIBI are interesting possibilities. Other possibilities involve the use of immune modulating substances such as lymphokines (e.g. IFN-γ, IL-2 and IL-12) or synthetic IFN-γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

The vaccine agent of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby immunogenic peptides, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, such as human serum albumin, are described Remington's Pharmaceutical Sciences (1980). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of one or more of the vaccine agents of the present invention, together with a suitable amount of a carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb one or more of the vaccine agents of the present invention. The controlled delivery may be exercised by selecting appropriate macromolecule (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, protamine, or sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate vaccine agents of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these vaccine agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, bu coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatine-microcapsules and

poly(methylmethacylate) microcapsules, respectively, orin colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The invention further provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine compositions of the invention.

Computer-Related Embodiments

As used herein the term "nucleic acid codes of the invention" encompass the nucleotide sequences comprising, consisting essentially of, or consisting of any one of the following: a) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 1: 1-70715, 70795-82207, 82297-83612, 83824-85297, 85418-86388, 86446-87495, 87523-88294, 88384-89483, 89650-92748, 97156-98309, 98476-99329, 99491-100026, 100212-100281, 100396-100538, 100682-100833, 100995-101920, 102087-102970, 103264-103724, and 103753-106746; b) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 70728, 87860, 88297, 94432, and 95340 of SEQ ID No 1; a nucleotide A at positions 82218, 83644, 83808, 87787, 87806, 94218, and 97144 of SEQ ID No 1; a nucleotide C at positions 87902, 88215, 88283, 92760, 93726, and 94422 of SEQ ID No 1; and a nucleotide T at positions 93903, and 94170 of SEQ ID No 1; c) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide G at positions 86435, 93592, 93680, 93681, 93682, 93728, 93761, and 95445 of SEQ ID No 1; a nucleotide A at positions 86434, 88355, 93240, 93471, and 93747of SEQ ID No 1; a nucleotide C at positions 93683, 95126, and 95444 of SEQ ID No 1; and a nucleotide T at positions 94154, and 94430 of SEQ ID No 1; d) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 1: 92975-92977, 93711-93715, 94151-94153, 94240-94243, 94770-94773, 94804-94808, 95121-95122, 95129-95135, 95148-95153, 95154-95159, 95173-95178, 95367-95374,

95410-95413, 95418-95420, 95430-95436, 95533-95535, and 95677-95677; e) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 2; f) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2471, and 5397 of SEQ ID No 2; a nucleotide C at positions 1013, 1979, and 2675 of SEQ ID No 2; a nucleotide G at positions 176, 749, 2685, 3593 of SEQ ID No 2; and a nucleotide T at positions 2156, and 2423 of SEQ ID No 2; g) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1493, 1724, and 2000; a nucleotide C at positions 1936, 3379, and 3697; a nucleotide G at positions 709, 1845, 1933, 1934, 1935, 1981, 2014, and 3698; and a nucleotide T at positions 2407, and 2683 of SEQ ID No 2; h) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 2: 1229-1231, 1964-1968, 2404-2406, 2493-2496, 3023-3026, 3057-3061, 3374-3375, 3382-3388, 3401-3406, 3407-3412, 3426-3431, 3620-3627, 3663-3666, 3671-3673, 3683-3689, 3786-3788 and 3930-3932; i) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 3: 1-162 and 747-872; j) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527, 2597, and 5523 of SEQ ID No 3; a nucleotide C at positions 1139, 2105, and 2801 of SEQ ID No 3; a nucleotide G at positions 176, 875, 2811, 3719 of SEQ ID No 3; and a nucleotide T at positions 2282, and 2549 of SEQ ID No 3; k) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708, 807, 1619, 1850, and 2126; a nucleotide C at positions 2062, 3505, and 3823; a nucleotide G at positions 709, 1971, 2059, 2060, 2061, 2107, 2140, and 3824; and a nucleotide T at positions 2533, and

2809 of SEQ ID No 3; l) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 3 or the complements thereof, wherein said contiguous span comprises nucleotide positions selected from the group consisting of the nucleotide positions of SEQ ID No 3: 1355-1357, 1892-1894, 2090-2094, 2530-2532, 2619-2622, 3149-3152, 3183-3187, 3500-3501, 3508-3514, 3527-3532, 3533-3538, 3552-3557, 3746-3749, 3789-3792, 3797-3799, 3809-3815, 3912-3914 and 4056-4058; m) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 4; n) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 253, 363, 527 and 2460 of SEQ ID No 4; a nucleotide C at position 1013 of SEQ ID No 4 and a nucleotide G at positions 176, and 749 of SEQ ID No 4; o) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least one nucleotide selected from the group consisting of a nucleotide A at positions 708 and 807 and a nucleotide G at position 709 of SEQ ID No 4; p) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises the pairs of nucleotide positions 1136-1137 of SEQ ID No 4; q) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 8 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 8: 1-500, 501-1000, 1001-1500, and 1501-1738; and, r) a nucleotide sequence complementary to any one of the preceding nucleotide sequences.

The "nucleic acid codes of the invention" further encompass nucleotide sequences homologous to: a) a contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 1: 1-70715, 70795-82207, 82297-83612, 83824-85297, 85418-86388, 86446-87495, 87523-88294, 88384-89483, 89650-92748, 97156-98309, 98476-99329, 99491-100026, 100212-100281, 100396-100538, 100682-100833, 100995-101920, 102087-102970, 103264-103724, and 103753-106746; b) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 2; c) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID

No 3 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 3: 1-162 and 747-872; d) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 4 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the nucleotide positions 1-162 of SEQ ID No 4; e) a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 8 or the complements thereof, wherein said contiguous span comprises at least 1, 2, 3, 5, or 10 of the following nucleotide positions of SEQ ID No 8: 1-500, 501-1000, 1001-1500, and 1501-1738; and f) sequences complementary to all of the preceding sequences. Homologous sequences refer to a sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, or 75% homology to these contiguous spans. Homology may be determined using any method described herein, including BLAST2N with the default parameters or with any modified parameters. Homologous sequences also may include RNA sequences in which uridines replace the thymines in the nucleic acid codes of the invention. It will be appreciated that the nucleic acid codes of the invention can be represented in the traditional single character format (See the inside back cover of Stryer, Lubert. *Biochemistry*, 3rd edition. W. H Freeman & Co., New York.) or in any other format or code which records the identity of the nucleotides in a sequence.

As used herein the term "polypeptide codes of the invention" encompass the polypeptide sequences comprising:

a) a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 5, wherein said contiguous span includes:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 5; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 5;

b) a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 6, wherein said contiguous span includes:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 245 in SEQ ID No 6; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at

amino acid position 55 and an arginine residue at amino acid position 225 in SEQ ID No 6; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exon 6bis, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 183-224 of the SEQ ID No 6;

c) a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 7, wherein said contiguous span includes:

i) a serine residue at amino acid position 170 and/or a lysine residue at amino acid position 203 in SEQ ID No 7; and/or

ii) at least one residue selected from the group consisting of a tyrosine residue at amino acid position 18, a cysteine residue at amino acid position 35, a methionine residue at amino acid position 55 and an arginine residue at amino acid position 183 in SEQ ID No 7; and/or

iii) at least 1, 2, 3, 5 or 10 of the amino acid encoded by the exons 9bis and 9ter, more particularly at least 1, 2, 3, 5 or 10 of the amino acid positions 313-368 of the SEQ ID No 7; and,

d) a contiguous span of at least 6 amino acids, preferably at least 8 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID No 9.

It will be appreciated that the polypeptide codes of the invention can be represented in the traditional single character format or three letter format (See the inside back cover of Stryer, Lubert. Biochemistry, 3rd edition. W. H Freeman & Co., New York.) or in any other format or code which records the identity of the polypeptides in a sequence.

It will be appreciated by those skilled in the art that the nucleic acid codes of the invention and polypeptide codes of the invention can be stored, recorded, and manipulated on any medium which can be read and accessed by a computer. As used herein, the words "recorded" and "stored" refer to a process for storing information on a computer medium. A skilled artisan can readily adopt any of the presently known methods for recording information on a computer readable medium to generate manufactures comprising one or more of the nucleic acid codes of the invention, or one or more of the polypeptide codes of the invention. Another aspect of the present invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, 20, 25, 30, or 50 nucleic acid codes of the invention. Another aspect of the present invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, 20, 25, 30, or 50 polypeptide codes of the invention.

Computer readable media include magnetically readable media, optically readable media, electronically readable media and magnetic/optical media. For example, the computer readable

media may be a hard disk, a floppy disk, a magnetic tape, CD-ROM, Digital Versatile Disk (DVD), Random Access Memory (RAM), or Read Only Memory (ROM) as well as other types of other media known to those skilled in the art.

Embodiments of the present invention include systems, particularly computer systems which store and manipulate the sequence information described herein. One example of a computer system 100 is illustrated in block diagram form in Figure 3. As used herein, "a computer system" refers to the hardware components, software components, and data storage components used to analyze the nucleotide sequences of the nucleic acid codes of the invention or the amino acid sequences of the polypeptide codes of the invention. In one embodiment, the computer system 100 is a Sun Enterprise 1000 server (Sun Microsystems, Palo Alto, CA). The computer system 100 preferably includes a processor for processing, accessing and manipulating the sequence data. The processor 105 can be any well-known type of central processing unit, such as the Pentium III from Intel Corporation, or similar processor from Sun, Motorola, Compaq or International Business Machines.

Preferably, the computer system 100 is a general purpose system that comprises the processor 105 and one or more internal data storage components 110 for storing data, and one or more data retrieving devices for retrieving the data stored on the data storage components. A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

In one particular embodiment, the computer system 100 includes a processor 105 connected to a bus which is connected to a main memory 115 (preferably implemented as RAM) and one or more internal data storage devices 110, such as a hard drive and/or other computer readable media having data recorded thereon. In some embodiments, the computer system 100 further includes one or more data retrieving device 118 for reading the data stored on the internal data storage devices 110.

The data retrieving device 118 may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, etc. In some embodiments, the internal data storage device 110 is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system 100 may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once inserted in the data retrieving device.

The computer system 100 includes a display 120 which is used to display output to a computer user. It should also be noted that the computer system 100 can be linked to other computer systems 125a-c in a network or wide area network to provide centralized access to the computer system 100.

Software for accessing and processing the nucleotide sequences of the nucleic acid codes of the invention or the amino acid sequences of the polypeptide codes of the invention (such as search tools, compare tools, and modeling tools etc.) may reside in main memory 115 during execution.

In some embodiments, the computer system 100 may further comprise a sequence comparer for comparing the above-described nucleic acid codes of the invention or the polypeptide codes of the invention stored on a computer readable medium to reference nucleotide or polypeptide sequences stored on a computer readable medium. A "sequence comparer" refers to one or more programs which are implemented on the computer system 100 to compare a nucleotide or polypeptide sequence with other nucleotide or polypeptide sequences and/or compounds including but not limited to peptides, peptidomimetics, and chemicals stored within the data storage means. For example, the sequence comparer may compare the nucleotide sequences of nucleic acid codes of the invention or the amino acid sequences of the polypeptide codes of the invention stored on a computer readable medium to reference sequences stored on a computer readable medium to identify homologies, motifs implicated in biological function, or structural motifs. The various sequence comparer programs identified elsewhere in this patent specification are particularly contemplated for use in this aspect of the invention.

Figure 4 is a flow diagram illustrating one embodiment of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database. The database of sequences can be a private database stored within the computer system 100, or a public database such as GENBANK, PIR OR SWISSPROT that is available through the Internet.

The process 200 begins at a start state 201 and then moves to a state 202 wherein the new sequence to be compared is stored to a memory in a computer system 100. As discussed above, the memory could be any type of memory, including RAM or an internal storage device.

The process 200 then moves to a state 204 wherein a database of sequences is opened for analysis and comparison. The process 200 then moves to a state 206 wherein the first sequence stored in the database is read into a memory on the computer. A comparison is then performed at a state 210 to determine if the first sequence is the same as the second sequence. It is important to note that this step is not limited to performing an exact comparison between the new sequence and the first sequence in the database. Well-known methods are known to those of skill in the art for comparing two nucleotide or protein sequences, even if they are not identical. For example, gaps can be introduced into one sequence in order to raise the homology level between the two tested sequences. The parameters that control whether gaps or other features are introduced into a sequence during comparison are normally entered by the user of the computer system.

Once a comparison of the two sequences has been performed at the state 210, a determination is made at a decision state 210 whether the two sequences are the same. Of course, the term "same" is not limited to sequences that are absolutely identical. Sequences that are within the homology parameters entered by the user will be marked as "same" in the process 200.

If a determination is made that the two sequences are the same, the process 200 moves to a state 214 wherein the name of the sequence from the database is displayed to the user. This state notifies the user that the sequence with the displayed name fulfills the homology constraints that were entered. Once the name of the stored sequence is displayed to the user, the process 200 moves to a decision state 218 wherein a determination is made whether more sequences exist in the database. If no more sequences exist in the database, then the process 200 terminates at an end state 220. However, if more sequences do exist in the database, then the process 200 moves to a state 224 wherein a pointer is moved to the next sequence in the database so that it can be compared to the new sequence. In this manner, the new sequence is aligned and compared with every sequence in the database.

It should be noted that if a determination had been made at the decision state 212 that the sequences were not homologous, then the process 200 would move immediately to the decision state 218 in order to determine if any other sequences were available in the database for comparison.

Accordingly, one aspect of the present invention is a computer system comprising a processor, a data storage device having stored thereon a nucleic acid code of the invention or a polypeptide code of the invention, a data storage device having retrievably stored thereon reference nucleotide sequences or polypeptide sequences to be compared to the nucleic acid code of the invention or polypeptide code of the invention and a sequence comparer for conducting the comparison. The sequence comparer may indicate a homology level between the sequences compared or identify structural motifs in the nucleic acid code of the invention and polypeptide codes of the invention or it may identify structural motifs in sequences which are compared to these nucleic acid codes and polypeptide codes. In some embodiments, the data storage device may have stored thereon the sequences of at least 2, 5, 10, 15, 20, 25, 30, or 50 of the nucleic acid codes of the invention or polypeptide codes of the invention.

(B3) Another aspect of the present invention is a method for determining the level of homology between a nucleic acid code of the invention and a reference nucleotide sequence, comprising the steps of reading the nucleic acid code and the reference nucleotide sequence through the use of a computer program which determines homology levels and determining homology between the nucleic acid code and the reference nucleotide sequence with the computer program. The computer program may be any of a number of computer programs for determining homology levels,

including those specifically enumerated herein, including BLAST2N with the default parameters or with any modified parameters. The method may be implemented using the computer systems described above. The method may also be performed by reading 2, 5, 10, 15, 20, 25, 30, or 50 of the above described nucleic acid codes of the invention through the use of the computer program and determining homology between the nucleic acid codes and reference nucleotide sequences.

Figure 5 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous. The process 250 begins at a start state 252 and then moves to a state 254 wherein a first sequence to be compared is stored to a memory. The second sequence to be compared is then stored to a memory at a state 256. The process 250 then moves to a state 260 wherein the first character in the first sequence is read and then to a state 262 wherein the first character of the second sequence is read. It should be understood that if the sequence is a nucleotide sequence, then the character would normally be either A, T, C, G or U. If the sequence is a protein sequence, then it should be in the single letter amino acid code so that the first and sequence sequences can be easily compared.

A determination is then made at a decision state 264 whether the two characters are the same. If they are the same, then the process 250 moves to a state 268 wherein the next characters in the first and second sequences are read. A determination is then made whether the next characters are the same. If they are, then the process 250 continues this loop until two characters are not the same. If a determination is made that the next two characters are not the same, the process 250 moves to a decision state 274 to determine whether there are any more characters either sequence to read.

If there aren't any more characters to read, then the process 250 moves to a state 276 wherein the level of homology between the first and second sequences is displayed to the user. The level of homology is determined by calculating the proportion of characters between the sequences that were the same out of the total number of sequences in the first sequence. Thus, if every character in a first 100 nucleotide sequence aligned with a every character in a second sequence, the homology level would be 100%.

Alternatively, the computer program may be a computer program which compares the nucleotide sequences of the nucleic acid codes of the present invention, to reference nucleotide sequences in order to determine whether the nucleic acid code of the invention differs from a reference nucleic acid sequence at one or more positions. Optionally such a program records the length and identity of inserted, deleted or substituted nucleotides with respect to the sequence of either the reference polynucleotide or the nucleic acid code of the invention. In one embodiment, the computer program may be a program which determines whether the nucleotide sequences of

the nucleic acid codes of the invention contain one or more single nucleotide polymorphisms (SNP) with respect to a reference nucleotide sequence. These single nucleotide polymorphisms may each comprise a single base substitution, insertion, or deletion.

Another aspect of the present invention is a method for determining the level of homology between a polypeptide code of the invention and a reference polypeptide sequence, comprising the steps of reading the polypeptide code of the invention and the reference polypeptide sequence through use of a computer program which determines homology levels and determining homology between the polypeptide code and the reference polypeptide sequence using the computer program.

Accordingly, another aspect of the present invention is a method for determining whether a nucleic acid code of the invention differs at one or more nucleotides from a reference nucleotide sequence comprising the steps of reading the nucleic acid code and the reference nucleotide sequence through use of a computer program which identifies differences between nucleic acid sequences and identifying differences between the nucleic acid code and the reference nucleotide sequence with the computer program. In some embodiments, the computer program is a program which identifies single nucleotide polymorphisms The method may be implemented by the computer systems described above and the method illustrated in Figure 5. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30, or 50 of the nucleic acid codes of the invention and the reference nucleotide sequences through the use of the computer program and identifying differences between the nucleic acid codes and the reference nucleotide sequences with the computer program.

In other embodiments the computer based system may further comprise an identifier for identifying features within the nucleotide sequences of the nucleic acid codes of the invention or the amino acid sequences of the polypeptide codes of the invention.

An "identifier" refers to one or more programs which identifies certain features within the above-described nucleotide sequences of the nucleic acid codes of the invention or the amino acid sequences of the polypeptide codes of the invention. In one embodiment, the identifier may comprise a program which identifies an open reading frame in the cDNAs codes of the invention.

Figure 6 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence. The process 300 begins at a start state 302 and then moves to a state 304 wherein a first sequence that is to be checked for features is stored to a memory 115 in the computer system 100. The process 300 then moves to a state 306 wherein a database of sequence features is opened. Such a database would include a list of each feature's attributes along with the name of the feature. For example, a feature name could be "Initiation Codon" and the attribute would be "ATG". Another example would be the feature

name "TAATAA Box" and the feature attribute would be "TAATAA". An example of such a database is produced by the University of Wisconsin Genetics Computer Group (www.gcg.com).

Once the database of features is opened at the state 306, the process 300 moves to a state 308 wherein the first feature is read from the database. A comparison of the attribute of the first feature with the first sequence is then made at a state 310. A determination is then made at a decision state 316 whether the attribute of the feature was found in the first sequence. If the attribute was found, then the process 300 moves to a state 318 wherein the name of the found feature is displayed to the user.

The process 300 then moves to a decision state 320 wherein a determination is made whether move features exist in the database. If no more features do exist, then the process 300 terminates at an end state 324. However, if more features do exist in the database, then the process 300 reads the next sequence feature at a state 326 and loops back to the state 310 wherein the attribute of the next feature is compared against the first sequence.

It should be noted, that if the feature attribute is not found in the first sequence at the decision state 316, the process 300 moves directly to the decision state 320 in order to determine if any more features exist in the database.

In another embodiment, the identifier may comprise a molecular modeling program which determines the 3-dimensional structure of the polypeptides codes of the invention. In some embodiments, the molecular modeling program identifies target sequences that are most compatible with profiles representing the structural environments of the residues in known three-dimensional protein structures. (See, e.g., Eisenberg et al., U.S. Patent No. 5,436,850 issued July 25, 1995, the disclosure of which is incorporated herein by reference in its entirety). In another technique, the known three-dimensional structures of proteins in a given family are superimposed to define the structurally conserved regions in that family. This protein modeling technique also uses the known three-dimensional structure of a homologous protein to approximate the structure of the polypeptide codes of the invention. (See e.g., Srinivasan, et al., U.S. Patent No. 5,557,535 issued September 17, 1996, the disclosure of which is incorporated herein by reference in its entirety). Conventional homology modeling techniques have been used routinely to build models of proteases and antibodies. (Sowdhamini et al., 1997). Comparative approaches can also be used to develop three-dimensional protein models when the protein of interest has poor sequence identity to template proteins. In some cases, proteins fold into similar three-dimensional structures despite having very weak sequence identities. For example, the three-dimensional structures of a number of helical cytokines fold in similar three-dimensional topology in spite of weak sequence homology.

The recent development of threading methods now enables the identification of likely folding patterns in a number of situations where the structural relatedness between target and template(s) is not detectable at the sequence level. Hybrid methods, in which fold recognition is performed using Multiple Sequence Threading (MST), structural equivalencies are deduced from the threading output using a distance geometry program DRAGON to construct a low resolution model, and a full-atom representation is constructed using a molecular modeling package such as QUANTA.

According to this 3-step approach, candidate templates are first identified by using the novel fold recognition algorithm MST, which is capable of performing simultaneous threading of multiple aligned sequences onto one or more 3-D structures. In a second step, the structural equivalencies obtained from the MST output are converted into interresidue distance restraints and fed into the distance geometry program DRAGON, together with auxiliary information obtained from secondary structure predictions. The program combines the restraints in an unbiased manner and rapidly generates a large number of low resolution model confirmations. In a third step, these low resolution model confirmations are converted into full-atom models and subjected to energy minimization using the molecular modeling package QUANTA. (See e.g., Aszódi et al., 1997).

The results of the molecular modeling analysis may then be used in rational drug design techniques to identify agents which modulate the activity of the polypeptide codes of the invention.

Accordingly, another aspect of the present invention is a method of identifying a feature within the nucleic acid codes of the invention or the polypeptide codes of the invention comprising reading the nucleic acid code(s) or the polypeptide code(s) through the use of a computer program which identifies features therein and identifying features within the nucleic acid code(s) or polypeptide code(s) with the computer program. In one embodiment, computer program comprises a computer program which identifies open reading frames. In a further embodiment, the computer program identifies structural motifs in a polypeptide sequence. In another embodiment, the computer program comprises a molecular modeling program. The method may be performed by reading a single sequence or at least 2, 5, 10, 15, 20, 25, 30, or 50 of the nucleic acid codes of the invention or the polypeptide codes of the invention through the use of the computer program and identifying features within the nucleic acid codes or polypeptide codes with the computer program.

The nucleic acid codes of the invention or the polypeptide codes of the invention may be stored and manipulated in a variety of data processor programs in a variety of formats. For example, they may be stored as text in a word processing file, such as MicrosoftWORD or

WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2, SYBASE, or ORACLE. In addition, many computer programs and databases may be used as sequence comparers, identifiers, or sources of reference nucleotide or polypeptide sequences to be compared to the nucleic acid codes of the invention or the polypeptide codes of the invention. The following list is intended not to limit the invention but to provide guidance to programs and databases which are useful with the nucleic acid codes of the invention or the polypeptide codes of the invention. The programs and databases which may be used include, but are not limited to: MacPattern (EMBL), DiscoveryBase (Molecular Applications Group), GeneMine (Molecular Applications Group), Look (Molecular Applications Group), MacLook (Molecular Applications Group), BLAST and BLAST2 (NCBI), BLASTN and BLASTX (Altschul et al, 1990), FASTA (Pearson and Lipman, 1988), FASTDB (Brutlag et al., 1990), Catalyst (Molecular Simulations Inc.), Catalyst/SHAPE (Molecular Simulations Inc.), Cerius^2.DBAccess (Molecular Simulations Inc.), HypoGen (Molecular Simulations Inc.), Insight II, (Molecular Simulations Inc.), Discover (Molecular Simulations Inc.), CHARMm (Molecular Simulations Inc.), Felix (Molecular Simulations Inc.), DelPhi, (Molecular Simulations Inc.), QuanteMM, (Molecular Simulations Inc.), Homology (Molecular Simulations Inc.), Modeler (Molecular Simulations Inc.), ISIS (Molecular Simulations Inc.), Quanta/Protein Design (Molecular Simulations Inc.), WebLab (Molecular Simulations Inc.), WebLab Diversity Explorer (Molecular Simulations Inc.), Gene Explorer (Molecular Simulations Inc.), SeqFold (Molecular Simulations Inc.), the EMBL/Swissprotein database, the MDL Available Chemicals Directory database, the MDL Drug Data Report data base, the Comprehensive Medicinal Chemistry database, Derwents's World Drug Index database, the BioByteMasterFile database, the Genbank database, and the Genseqn database. Many other programs and data bases would be apparent to one of skill in the art given the present disclosure.

Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

Throughout this application, various publications, patents and published patent applications are cited. The disclosures of these publications, patents and published patent specification referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the sate of the art to which this invention pertains.

EXAMPLES

Example 1

Detection Of PCTA-1 Biallelic Markers: DNA Extraction

Blood donors were from French Caucasian origin. They presented a sufficient diversity for being representative of a French heterogeneous population. The DNA from 100 unrelated and healthy individuals was extracted, pooled and tested for the detection of biallelic markers. The pool was constituted by mixing equivalent quantities of DNA from each individual.

30 ml of peripheral venous blood were taken from each donor in the presence of EDTA. Cells (pellet) were collected after centrifugation for 10 minutes at 2000 rpm. Red cells were lysed by a lysis solution (50 ml final volume : 10 mM Tris pH7.6; 5 mM $MgCl_2$; 10 mM NaCl). The solution was centrifuged (10 minutes, 2000 rpm) as many times as necessary to eliminate the residual red cells present in the supernatant, after resuspension of the pellet in the lysis solution.

The pellet of white cells was lysed overnight at 42°C with 3.7 ml of lysis solution composed of:

- 3 ml TE 10-2 (Tris-HCl 10 mM, EDTA 2 mM) / NaCl 0.4 M
- 200 µl SDS 10%
- 500 µl K-proteinase (2 mg K-proteinase in TE 10-2 / NaCl 0.4 M).

For the extraction of proteins, 1 ml saturated NaCl (6M) (1/3.5 v/v) was added. After vigorous agitation, the solution was centrifuged for 20 minutes at 10000 rpm.

For the precipitation of DNA, 2 to 3 volumes of 100% ethanol were added to the previous supernatant, and the solution was centrifuged for 30 minutes at 2000 rpm. The DNA solution was rinsed three times with 70% ethanol to eliminate salts, and centrifuged for 20 minutes at 2000 rpm. The pellet was dried at 37°C, and resuspended in 1 ml TE 10-1 or 1 ml water. The DNA concentration was evaluated by measuring the OD at 260 nm (1 unit OD = 50 µg/ml DNA).

To determine the presence of proteins in the DNA solution, the OD 260 / OD 280 ratio was determined. Only DNA preparations having a OD 260 / OD 280 ratio between 1.8 and 2 were used in the subsequent examples described below.

Example 2

Detection Of The Biallelic Markers: Amplification Of Genomic DNA By PCR

The amplification of specific genomic sequences of the DNA samples of example 1 was carried out on the pool of DNA obtained previously. In addition, 10 individual samples were similarly amplified.

PCR assays were performed using the following protocol:

Final volume	25 µl
DNA	2 ng/µl
$MgCl_2$	2 mM
dNTP (each)	200 µM
primer (each)	2.9 ng/µl
Ampli Taq Gold DNA polymerase	0.05 unit/µl
PCR buffer (10x = 0.1 M TrisHCl pH8.3 0.5M KCl	1x

Each pair of primers was designed using the sequence information of our total genomic sequence (SEQ ID No 1) and the OSP software (Hillier & Green, 1991). These primers had about 20 nucleotides in length and their respective sequences are disclosed in Table 1 and had the sequences disclosed in Table 1 in the columns labeled "Position range of amplification primer in SEQ ID No 1" and "Complementary position range of amplification primer in SEQ ID No 1".

The primers contained a common oligonucleotide tail upstream of the specific bases targeted for amplification which was useful for sequencing.

Primers from the columns labeled "Position range of amplification primer in SEQ ID No 1," contain the following additional PU 5' sequence: TGTAAAACGACGGCCAGT; and primers from the columns labeled "Complementary position range of amplification primer in SEQ ID No 1," contain the following RP 5' sequence: CAGGAAACAGCTATGACC. The primer containing the additional PU 5' sequence is listed in SEQ ID No 10. The primer containing the additional RP 5' sequence is listed in SEQ ID No 11.

The synthesis of these primers was performed following the phosphoramidite method, on a GENSET UFPS 24.1 synthesizer.

DNA amplification was performed on a Genius II thermocycler. After heating at 94°C for 10 min, 40 cycles were performed. Each cycle comprised: 30 sec at 94°C, 55°C for 1 min, and 30 sec at 72°C. For final elongation, 7 min at 72°C end the amplification. The quantities of the amplification products obtained were determined on 96-well microtiter plates, using a fluorometer and Picogreen as intercalant agent (Molecular Probes).

Table 1

Amplicon	Position range of the amplicon in SEQ ID 1		Primer name	Position range of amplification primer in SEQ ID No 1		primer name	Complementary position range of amplification primer in SEQ ID No 1	
99-1601	1	506	B1	1	18	C1	486	506
99-13801	2607	3054	B2	2607	2627	C2	3035	3054
99-13806	11883	12331	B3	11883	11902	C3	12313	12331
99-13799	12379	12909	B4	12379	12399	C4	12889	12909
99-13798	17442	17887	B5	17442	17462	C5	17868	17887
99-1602	21881	22506	B6	21881	21899	C6	22487	22506
99-13794	28669	29149	B7	28669	28689	C7	29131	29149
Amplicon	**Position range of the amplicon in SEQ ID 1**		**Primer name**	**Position range of amplification primer in SEQ ID No 1**		**primer name**	**Complementary position range of amplification primer in SEQ ID No 1**	
99-13812	30941	31457	B8	30941	30961	C8	31437	31457
99-13805	31560	32075	B9	31560	31579	C9	32057	32075
99-1587	34515	34909	B10	34515	34535	C10	34890	34909
99-1582	45325	46018	B11	45325	45343	C11	46000	46018
99-1585	49765	50310	B12	49765	49784	C12	50291	50310
99-1607	54726	55325	B13	54726	54746	C13	55307	55325
99-1577	64135	64536	B14	64135	64153	C14	64518	64536
99-1591	65202	65834	B15	65202	65219	C15	65815	65834
99-1572	66653	67295	B16	66653	66671	C16	67275	67295
5-169	67627	68043	B17	67627	67646	C17	68024	68043
5-264	67246	67696	B18	67246	67263	C18	67678	67696
5-170	67977	68424	B19	67977	67994	C19	68406	68424
5-171	68322	68742	B20	68322	68340	C20	68725	68742
5-1	70507	70928	B21	70507	70524	C21	70909	70928
99-1578	79940	80575	B22	79940	79957	C22	80557	80575
99-1605	82057	82504	B23	82057	82077	C23	82484	82504
5-2	82058	82492	B24	82058	82077	C24	82473	82492
5-3	83561	83982	B25	83561	83578	C25	83965	83982
5-4	83597	84017	B26	83597	83616	C26	83999	84017
5-260	83793	84167	B27	83793	83812	C27	84148	84167
5-9	85153	85576	B28	85153	85170	C28	85559	85576
5-5	86239	86539	B29	86239	86257	C29	86519	86539
5-202	87619	88050	B30	87619	87638	C30	88033	88050
5-7	88104	88536	B31	88104	88122	C31	88519	88536
5-181	89338	89758	B32	89338	89357	C32	89739	89758
5-10	92722	93142	B33	92722	92741	C33	93124	93142
5-11	93090	93509	B34	93090	93108	C34	93490	93509
5-12	93460	93881	B35	93460	93478	C35	93862	93881
5-13	93759	94192	B36	93759	93776	C36	94175	94192
5-14	94127	94554	B37	94127	94144	C37	94535	94554
5-15	94504	94921	B38	94504	94521	C38	94904	94921
5-16	94833	95251	B39	94833	94850	C39	95232	95251
5-17	95124	95561	B40	95124	95142	C40	95542	95561

5-18	95290	95708	B41	95290	95308	C41	95689	95708
5-300	95533	95952	B42	95533	95551	C42	95934	95952
5-262	96097	96591	B43	96097	96115	C43	96574	96591
5-263	96548	97001	B44	96548	96565	C44	96982	97001
5-265	96901	97309	B45	96901	96918	C45	97292	97309
99-7183	102156	102604	B46	102156	102176	C46	102584	102604
99-7207	105570	106074	B47	105570	105588	C47	106056	106074

Example 3

Detection Of The Biallelic Markers: Sequencing Of Amplified Genomic DNA And Identification Of Polymorphisms

The sequencing of the amplified DNA obtained in example 2 was carried out on ABI 377 sequencers. The sequences of the amplification products were determined using automated dideoxy terminator sequencing reactions with a dye terminator cycle sequencing protocol. The products of the sequencing reactions were run on sequencing gels and the sequences were determined using gel image analysis (ABI Prism DNA Sequencing Analysis software (2.1.2 version)).

The sequence data were further evaluated using the above mentioned polymorphism analysis software designed to detect the presence of biallelic markers among the pooled amplified fragments. The polymorphism search was based on the presence of superimposed peaks in the electrophoresis pattern resulting from different bases occurring at the same position as described previously.

47 fragments of amplification were analyzed. In these segments, 125 markers were detected. The localization of the biallelic markers was as shown in Table 2. Table 3 comprises the polynucleotides defining the *PCTA-1*-related biallelic markers. They could be used as probes and their sequence are disclosed in Table 3 in "Position range of probes in SEQ ID No 1".

Table 2

Amplicon	BM	Marker Name	Localization in *PCTA-1* gene	Polymorphism (frequency %)		BM position in SEQ ID			
				all1	all2	No 1	No 2	No 3	No 4
99-1601	A1	99-1601-278	5'regulatory	A	C	278			
99-1601	A2	99-1601-402	5'regulatory	A (66)	T	402			
99-1601	A3	99-1601-472	5'regulatory	A	T	472			
99-13801	A4	99-13801-100	5'regulatory	T	C	2955			
99-13806	A5	99-13806-166	5'regulatory	G	A	12167			
99-13799	A6	99-13799-376	5'regulatory	T	G	12536			
99-13798	A7	99-13798-297	5'regulatory	T	C	17593			

99-13798	A8	99-13798-284	5'regulatory	T	C	17606			
99-1602	A9	99-1602-200	5'regulatory	C	G	22079			
99-13794	A10	99-13794-186	5'regulatory	T	C	28964			
99-13794	A11	99-13794-147	5'regulatory	C	G	29003			
99-13812	A12	99-13812-384	5'regulatory	T	C	31077			
99-13805	A13	99-13805-313	5'regulatory	T	C	31766			
99-1587	A14	99-1587-281	5'regulatory	A	G	34791			
99-1582	A15	99-1582-430	5'regulatory	C	T	45751			
99-1585	A16	99-1585-465	5'regulatory	T	C	49847			
99-1585	A17	99-1585-457	5'regulatory	T	C	49855			
99-1585	A18	99-1585-426	5'regulatory	G	A	49886			
99-1585	A19	99-1585-412	5'regulatory	G	A	49900			
99-1585	A20	99-1585-406	5'regulatory	C	A	49906			
99-1585	A21	99-1585-391	5'regulatory	C	A	49921			
99-1585	A22	99-1585-373	5'regulatory	G	A	49939			
99-1585	A23	99-1585-55	5'regulatory	C	A	50256			
99-1607	A24	99-1607-373	5'regulatory	T	C	54955			
99-1577	A25	99-1577-105	5'regulatory	A (54)	G	64239			
99-1591	A26	99-1591-235	5'regulatory	A	G	65436			
99-1591	A27	99-1591-295	5'regulatory	G	T	65496			
99-1572	A28	99-1572-315	Promoter	C	T	66967			
99-1572	A29	99-1572-335	Promoter	A	G	66987			
99-1572	A30	99-1572-440	Promoter	C (32)	T	67092			
99-1572	A31	99-1572-477	Promoter	A	T	67129			
99-1572	A32	99-1572-578	Promoter	C	T	67229			
5-264	A33	5-264-188	Promoter	A	G	67433			
5-169	A34	5-169-97	Promoter	G (18)	C	67723			
5-169	A35	5-169-208	Promoter	A (<1)	G	67834			
5-169	A36	5-169-331	Promoter	C (99)	T	67955			
5-170	A37	5-170-238	Promoter	A	G	68213			
5-170	A38	5-170-288	Promoter	A (1)	C	68263			
5-170	A39	5-170-400	Promoter	G	C	68375			
5-171	A40	5-171-156	Promoter	G	T	68477			
5-171	A41	5-171-204	Promoter	C (30)	T	68525			
5-171	A42	5-171-273	Promoter	A	G	68594			
5-171	A43	5-171-289	Promoter	C	T	68610			
5-1	A44	5-1-60	Intron 0	C (1)	T	70566			
5-1	A45	5-1-222	Exon 1	A	G	70728	176	176	176
99-1578	A46	99-1578-99	Intron 1	G	T	80038			
99-1578	A47	99-1578-179	Intron 1	A	T	80118			
99-1578	A48	99-1578-231	Intron 1	Ins AC		80170			
99-1578	A49	99-1578-245	Intron 1	del AT		80183			
99-1578	A50	99-1578-496	Intron 1	C	T	80435			
5-2	A51	5-2-30	Intron 1	Ins CAG		82090			
5-2	A52	5-2-109	Intron 1	G	T	82165			
5-2	A53	5-2-113	Intron 1	Del GTTT		82169			
5-2	A54	5-2-162	Exon 2	A (67)	T	82218	253	253	253

5-2	A55	5-2-178	Exon 2	C (67)	T	82234	269	269	269
5-2	A56	5-2-213	Exon 2	C (33)	T	82268	303	303	303
99-1605	A57	99-1605-112	Intron 2	T (67)	C	82393			
5-3	A58	5-3-27	Intron 2	A	G	83587			
5-3	A59	5-3-83	Exon 3	C (39)	T	83643	362	362	362
5-3	A60	5-3-84	Exon 3	A (29)	G	83644	363	363	363
5-3	A61	5-3-248	Exon 3	A	G	83808	527	527	527
5-3	A62	5-3-321	Intron 3	G	T	83881			
5-3	A63	5-3-324	Intron 3	C	T	83884			
5-4	A64	5-4-313	Intron 3	A	G	83909			
5-3	A65	5-3-377	Intron 3	ins TTTG		83937			
5-4	A66	5-4-351	Intron 3	C	T	83947			
5-4	A67	5-4-386	Intron 3	A	G	83982			
5-4	A68	5-4-392	Intron 3	GGG	TA	83988			
5-260	A69	5-260-255	Intron 3	C	T	84047			
5-260	A70	5-260-300	Intron 3	C	T	84092			
5-260	A71	5-260-353	Intron 3	C	T	84145			
5-9	A72	5-9-50	Intron 3	C	T	85202			
5-5	A73	5-5-21	Intron 4	A	G	86259			
5-5	A74	5-5-85	Intron 4	TATA AAAT ATT	ACAG GTTA TATA	86323			
5-202	A75	5-202-95	Exon 6bis	G	T (<1)	87713		810	
5-202	A76	5-202-117	Exon 6bis	A (<1)	T	87735		832	
5-202	A77	5-202-169	Intron 6bis	A	C	87787			
5-202	A78	5-202-188	Intron 6bis	A	G	87806			
5-202	A79	5-202-242	Intron 6bis	A	G	87860			
5-202	A80	5-202-284	Intron 6bis	C	T	87902			
5-202	A81	5-202-362	Intron 6bis	del CC		87980			
5-202	A82	5-202-394	Intron 6bis	C	T	88012			
5-7	A83	5-7-113	Intron 6bis	C	T	88215			
5-7	A84	5-7-181	Intron 6bis	G	C	88283			
5-7	A85	5-7-195	Exon 7	G (25)	C	88297	749	875	749
5-7	A86	5-7-340	Intron 7	C	T	88442			
5-7	A87	5-7-369	Intron 7	A	T	88471			
5-7	A88	5-7-378	Intron 7	C	T	88480			
5-181	A89	5-181-57	Intron 7	A	G	89394			
5-181	A90	5-181-127	Intron 7	C	T	89464			
5-181	A91	5-181-134	Intron 7	C	T	89471			
5-181	A92	5-181-321	Intron 8	A	C	89658			
5-10	A93	5-10-39	Exon 9 exon 9bis	C	T	92760	1013	1139	1013
5-10	A94	5-10-302	Exon 9 Intron 9bis	A	G	93023	1276	1402	
5-10	A95	5-10-334	Exon 9 Intron 9bis	A	C	93055	1308	1434	
5-11	A96	5-11-158	Exon 9 Intron 9bis	A (22)	G	93247	1500	1626	

5-11	A97	5-11-230	Exon 9 Intron 9bis	G	T	93319	1572	1698	
5-11	A98	5-11-234	Exon 9 Intron 9bis	C	T	93323	1576	1702	
5-11	A99	5-11-299	Exon 9 Intron 9bis	A	T	93388	1641	1767	
5-11	A100	5-11-304	Exon 9 Intron 9bis	A	C	93393	1646	1772	
5-11	A101	5-11-329	Exon 9 Intron 9bis	C	T	93418	1671	1797	
5-12	A102	5-12-56	Exon 9 Intron 9bis	ins CTTT		93515	1768	1894	
5-12	A103	5-12-267	Exon 9 Intron 9bis	A	C	93726	1979	2105	
5-13	A104	5-13-145	Exon 9 Intron 9bis	C	T	93903	2156	2282	
5-14	A105	5-14-44	Exon 9 Intron 9bis	C	T	94170	2423	2549	
5-14	A106	5-14-93	Exon 9 Intron 9bis	A	T	94218	2471	2597	
5-14	A107	5-14-144	Exon 9 Intron 9bis	ins T		94269	2522	2648	
5-14	A108	5-14-165	Exon 9 Intron 9bis	C	T	94290	2543	2669	
5-14	A109	5-14-297	Exon 9 Intron 9bis	A	C	94422	2675	2801	
5-14	A110	5-14-307	Exon 9 Intron 9bis	G	T	94432	2685	2811	
5-15	A111	5-15-219	Exon 9 Intron 9bis	A	T	94720	2973	3099	
5-16	A112	5-16-157	Exon 9 Intron 9bis	A	G	94989	3242	3368	
5-17	A113	5-17-140	Exon 9 Intron 9bis	A	G	95261	3514	3640	
5-18	A114	5-18-51	Exon 9 Intron 9bis	G	T	95340	3593	3719	
5-18	A115	5-18-208	Exon 9 Intron 9bis	A	C	95497	3750	3876	
5-300	A116	5-300-238	Exon 9 Intron 9bis	C	T	95770	4023	4149	
5-300	A117	5-300-287	Exon 9 Intron 9bis	A	G	95819	4072	4198	
5-262	A118	5-262-49	Exon 9 Exon 9ter	ins C		96145	4398	4524	1461
5-262	A119	5-262-85	Exon 9 Exon 9ter	C	T	96181	4434	4560	1497
5-262	A120	5-262-254	Exon 9 Exon 9ter	C	T	96350	4603	4729	1666
5-263	A121	5-263-404	Exon 9 Exon 9ter	C	T	96951	5204	5330	2267

5-265	A122	5-265-244	Exon 9 Exon 9ter	A	G	97144	5397	5523	2460
5-265	A123	5-265-376	3'regualtory	A	G	97276			
99-7183	A124	99-7183-338	3'regualtory	C	T	102267			
99-7207	A125	99-7207-138	3'regualtory	A	G	105937			

BM refers to "biallelic marker". All1 and all2 refer respectively to allele 1 and allele 2 of the biallelic marker. "Frequency%" refers to the frequency of the allele in percentage in control population. Frequencies corresponded to a population of random blood donors of French Caucasian origin.

Table 3

BM	Marker Name	Position range of probes in SEQ ID No 1		Probes
A1	99-1601-278	255	301	P1
A2	99-1601-402	379	425	P2
A3	99-1601-472	449	495	P3
A4	99-13801-100	2932	2978	P4
A5	99-13806-166	12144	12190	P5
A6	99-13799-376	12513	12559	P6
A7	99-13798-297	17570	17616	P7
A8	99-13798-284	17583	17629	P8
A9	99-1602-200	22056	22102	P9
A10	99-13794-186	28941	28987	P10
A11	99-13794-147	28980	29026	P11
A12	99-13812-384	31054	31100	P12
A13	99-13805-313	31743	31789	P13
A14	99-1587-281	34768	34814	P14
A15	99-1582-430	45728	45774	P15
A16	99-1585-465	49824	49870	P16
A17	99-1585-457	49832	49878	P17
A18	99-1585-426	49863	49909	P18
A19	99-1585-412	49877	49923	P19
A20	99-1585-406	49883	49929	P20
A21	99-1585-391	49898	49944	P21
A22	99-1585-373	49916	49962	P22
A23	99-1585-55	50233	50279	P23
A24	99-1607-373	54932	54978	P24
A25	99-1577-105	64216	64262	P25
A26	99-1591-235	65413	65459	P26
A27	99-1591-295	65473	65519	P27
A28	99-1572-315	66944	66990	P28
A29	99-1572-335	66964	67010	P29
A30	99-1572-440	67069	67115	P30
A31	99-1572-477	67106	67152	P31

A32	99-1572-578	67206	67252	P32
A33	5-264-188	67410	67456	P33
A34	5-169-97	67700	67746	P34
A35	5-169-208	67811	67857	P35
A36	5-169-331	67932	67978	P36
A37	5-170-238	68190	68236	P37
A38	5-170-288	68240	68286	P38
A39	5-170-400	68352	68398	P39
A40	5-171-156	68454	68500	P40
A41	5-171-204	68502	68548	P41
A42	5-171-273	68571	68617	P42
A43	5-171-289	68587	68633	P43
A44	5-1-60	70543	70589	P44
A45	5-1-222	70705	70751	P45
A46	99-1578-99	80015	80061	P46
A47	99-1578-179	80095	80141	P47
A48	99-1578-231	80147	80193	P48
A49	99-1578-245	80160	80206	P49
A50	99-1578-496	80412	80458	P50
A51	5-2-30	82067	82113	P51
A52	5-2-109	82142	82188	P52
A53	5-2-113	82146	82192	P53
A54	5-2-162	82195	82241	P54
A55	5-2-178	82211	82257	P55
A56	5-2-213	82245	82291	P56
A57	99-1605-112	82370	82416	P57
A58	5-3-27	83564	83610	P58
A59	5-3-83	83620	83666	P59
A60	5-3-84	83621	83667	P60
A61	5-3-248	83785	83831	P61
A62	5-3-321	83858	83904	P62
A63	5-3-324	83861	83907	P63
A64	5-4-313	83886	83932	P64
A65	5-3-377	83914	83960	P65
A66	5-4-351	83924	83970	P66
A67	5-4-386	83959	84005	P67
A68	5-4-392	83965	84011	P68
A69	5-260-255	84024	84070	P69
A70	5-260-300	84069	84115	P70
A71	5-260-353	84122	84168	P71
A72	5-9-50	85179	85225	P72
A73	5-5-21	86236	86282	P73
A74	5-5-85	86300	86346	P74
A75	5-202-95	87690	87736	P75
A76	5-202-117	87712	87758	P76
A77	5-202-169	87764	87810	P77
A78	5-202-188	87783	87829	P78
A79	5-202-242	87837	87883	P79
A80	5-202-284	87879	87925	P80

A81	5-202-362	87957	88003	P81
A82	5-202-394	87989	88035	P82
A83	5-7-113	88192	88238	P83
A84	5-7-181	88260	88306	P84
A85	5-7-195	88274	88320	P85
A86	5-7-340	88419	88465	P86
A87	5-7-369	88448	88494	P87
A88	5-7-378	88457	88503	P88
A89	5-181-57	89371	89417	P89
A90	5-181-127	89441	89487	P90
A91	5-181-134	89448	89494	P91
A92	5-181-321	89635	89681	P92
A93	5-10-39	92737	92783	P93
A94	5-10-302	93000	93046	P94
A95	5-10-334	93032	93078	P95
A96	5-11-158	93224	93270	P96
A97	5-11-230	93296	93342	P97
A98	5-11-234	93300	93346	P98
A99	5-11-299	93365	93411	P99
A100	5-11-304	93370	93416	P100
A101	5-11-329	93395	93441	P101
A102	5-12-56	93492	93538	P102
A103	5-12-267	93703	93749	P103
A104	5-13-145	93880	93926	P104
A105	5-14-44	94147	94193	P105
A106	5-14-93	94195	94241	P106
A107	5-14-144	94246	94292	P107
A108	5-14-165	94267	94313	P108
A109	5-14-297	94399	94445	P109
A110	5-14-307	94409	94455	P110
A111	5-15-219	94697	94743	P111
A112	5-16-157	94966	95012	P112
A113	5-17-140	95238	95284	P113
A114	5-18-51	95317	95363	P114
A115	5-18-208	95474	95520	P115
A116	5-300-238	95747	95793	P116
A117	5-300-287	95796	95842	P117
A118	5-262-49	96122	96168	P118
A119	5-262-85	96158	96204	P119
A120	5-262-254	96327	96373	P120
A121	5-263-404	96928	96974	P121
A122	5-265-244	97121	97167	P122
A123	5-265-376	97253	97299	P123
A124	99-7183-338	102244	102290	P124
A125	99-7207-138	105914	105960	P125

Example 4

Validation Of The Polymorphisms Through Microsequencing

The biallelic markers identified in example 3 were further confirmed and their respective frequencies were determined through microsequencing. Microsequencing was carried out for each individual DNA sample described in Example 1.

Amplification from genomic DNA of individuals was performed by PCR as described above for the detection of the biallelic markers with the same set of PCR primers (Table 1).

The preferred primers used in microsequencing had about 19 nucleotides in length and hybridized just upstream of the considered polymorphic base. Their sequences are disclosed in Table 4 in columns labeled " Position range of microsequencing primer mis. 1 in SEQ ID No 1" and " Complementary position range of microsequencing primer mis. 2 in SEQ ID No 1".

Mis 1 and Mis 2 respectively refer to microsequencing primers which hybridized with the non-coding strand of the *PCTA-1* gene or with the coding strand of the *PCTA-1* gene.

The microsequencing reaction was performed as follows :

10 µl of PCR products were added to 20 µl of microsequencing reaction mixture containing : 10 pmol microsequencing oligonucleotide (crude synthesis, 5 OD), 1 U Thermosequenase (Amersham E79000G), 1.25 µl Thermosequenase buffer (260 mM Tris HCl pH 9.5, 65 mM $MgCl_2$), and the appropriate fluorescent ddNTPs complementary to the nucleotides at the polymorphic site corresponding to the polymorphic bases (11.25 nM TAMRA-ddTTP ; 16.25 nM ROX-ddCTP ; 1.675 nM REG-ddATP ; 1.25 nM RHO-ddGTP ; Perkin Elmer, Dye Terminator Set 401095). After 4 minutes at 94°C, 20 PCR cycles of 15 sec at 55°C, 5 sec at 72°C, and 10 sec at 94°C were carried out in a thermocycler. After amplification, the unincorporated dye terminators were removed by ethanol precipitation. After discarding the supernatants, the microplate was evaporated to dryness under reduced pressure (Speed Vac) ; samples were resuspended in 2.5 µl formamide EDTA loading buffer and heated for 2 min at 95°C. 0.8 µl microsequencing reaction were loaded on a 10 % (19:1) polyacrylamide sequencing gel. The data were collected by an ABI PRISM 377 DNA sequencer and processed using the GENESCAN software (Perkin Elmer).

Following gel analysis, data were automatically processed with software that allows the determination of the alleles of biallelic markers present in each amplified fragment.

The software evaluates such factors as whether the intensities of the signals resulting from the above microsequencing procedures are weak, normal, or saturated, or whether the signals are ambiguous. In addition, the software identifies significant peaks (according to shape and height criteria). Among the significant peaks, peaks corresponding to the targeted site are identified based on their position. When two significant peaks are detected for the same

position, each sample is categorized classification as homozygous or heterozygous type based on the height ratio.

Table 4

Marker Name	BM	Mis. 1	Position range of microsequencing primer mis. 1 in SEQ ID No 1		Mis. 2	Complementary position range of microsequencing primer mis. 2 in SEQ ID No 1	
99-1601-278	A1	D1	258	277	E1	279	298
99-1601-402	A2	**D2**	382	401	E2	403	422
99-1601-472	A3	D3	452	471	E3	473	492
99-13801-100	A4	D4	2935	2954	E4	2956	2975
99-13806-166	A5	D5	12147	12166	E5	12168	12187
99-13799-376	A6	D6	12516	12535	E6	12537	12556
99-13798-297	A7	D7	17573	17592	E7	17594	17613
99-13798-284	A8	D8	17586	17605	E8	17607	17626
99-1602-200	A9	D9	22059	22078	E9	22080	22099
99-13794-186	A10	D10	28944	28963	E10	28965	28984
99-13794-147	A11	D11	28983	29002	**E11**	29004	29023
99-13812-384	A12	D12	31057	31076	E12	31078	31097
99-13805-313	A13	D13	31746	31765	E13	31767	31786
99-1587-281	A14	D14	34771	34790	**E14**	34792	34811
99-1582-430	A15	**D15**	45731	45750	E15	45752	45771
99-1585-465	A16	D16	49827	49846	E16	49848	49867
99-1585-457	A17	D17	49835	49854	E17	49856	49875
99-1585-426	A18	D18	49866	49885	E18	49887	49906
99-1585-412	A19	D19	49880	49899	E19	49901	49920
99-1585-406	A20	D20	49886	49905	E20	49907	49926
99-1585-391	A21	D21	49901	49920	E21	49922	49941
99-1585-373	A22	D22	49919	49938	**E22**	49940	49959
99-1585-55	A23	D23	50236	50255	E23	50257	50276
99-1607-373	A24	**D24**	54935	54954	E24	54956	54975
99-1577-105	A25	D25	64219	64238	**E25**	64240	64259
99-1591-235	A26	D26	65416	65435	**E26**	65437	65456
99-1591-295	A27	D27	65476	65495	E27	65497	65516
99-1572-315	A28	D28	66947	66966	E28	66968	66987
99-1572-335	A29	D29	66967	66986	E29	66988	67007
99-1572-440	A30	**D30**	67072	67091	E30	67093	67112
99-1572-477	A31	D31	67109	67128	E31	67130	67149
99-1572-578	A32	D32	67209	67228	E32	67230	67249
5-264-188	A33	D33	67413	67432	E33	67434	67453
5-169-97	A34	**D34**	67703	67722	E34	67724	67743
5-169-208	A35	D35	67814	67833	**E35**	67835	67854
5-169-331	A36	**D36**	67935	67954	E36	67956	67975
5-170-238	A37	D37	68193	68212	E37	68214	68233
5-170-288	A38	**D38**	68243	68262	E38	68264	68283
5-170-400	A39	D39	68355	68374	E39	68376	68395


5-171-156	A40	D40	68457	68476	E40	68478	68497
5-171-204	A41	**D41**	68505	68524	E41	68526	68545
5-171-273	A42	D42	68574	68593	**E42**	68595	68614
5-171-289	A43	D43	68590	68609	E43	68611	68630
5-1-60	A44	**D44**	70546	70565	E44	70567	70586
5-1-222	A45	D45	70708	70727	E45	70729	70748
99-1578-99	A46	D46	80018	80037	E46	80039	80058
99-1578-179	A47	D47	80098	80117	E47	80119	80138
99-1578-231	A48	D48	80150	80169	E48	80171	80190
99-1578-245	A49	D49	80163	80182	E49	80184	80203
99-1578-496	A50	**D50**	80415	80434	E50	80436	80455
5-2-30	A51	D51	82070	82089	E51	82091	82110
5-2-109	A52	D52	82145	82164	**E52**	82166	82185
5-2-113	A53	**D53**	82149	82168	**E53**	82170	82189
5-2-162	A54	**D54**	82198	82217	E54	82219	82238
5-2-178	A55	D55	82214	82233	**E55**	82235	82254
5-2-213	A56	**D56**	82248	82267	**E56**	82269	82288
99-1605-112	A57	**D57**	82373	82392	E57	82394	82413
5-3-27	A58	D58	83567	83586	E58	83588	83607
5-3-83	A59	**D59**	83623	83642	E59	83644	83663
5-3-84	A60	D60	83624	83643	**E60**	83645	83664
5-3-248	A61	D61	83788	83807	**E61**	83809	83828
5-3-321	A62	D62	83861	83880	E62	83882	83901
5-3-324	A63	D63	83864	83883	E63	83885	83904
5-4-313	A64	D64	83889	83908	**E64**	83910	83929
5-3-377	A65	D65	83917	83936	E65	83938	83957
5-4-351	A66	D66	83927	83946	E66	83948	83967
5-4-386	A67	D67	83962	83981	E67	83983	84002
5-4-392	A68	D68	83968	83987	E68	83989	84008
5-260-255	A69	D69	84027	84046	E69	84048	84067
5-260-300	A70	D70	84072	84091	E70	84093	84112
5-260-353	A71	D71	84125	84144	E71	84146	84165
5-9-50	A72	D72	85182	85201	E72	85203	85222
5-5-21	A73	D73	86239	86258	**E73**	86260	86279
5-5-85	A74	D74	86303	86322	E74	86324	86343
5-202-95	A75	D75	87693	87712	**E75**	87714	87733
5-202-117	A76	**D76**	87715	87734	E76	87736	87755
5-202-169	A77	D77	87767	87786	E77	87788	87807
5-202-188	A78	D78	87786	87805	E78	87807	87826
5-202-242	A79	D79	87840	87859	E79	87861	87880
5-202-284	A80	D80	87882	87901	E80	87903	87922
5-202-362	A81	D81	87960	87979	E81	87981	88000
5-202-394	A82	D82	87992	88011	E82	88013	88032
5-7-113	A83	D83	88195	88214	E83	88216	88235
5-7-181	A84	D84	88263	88282	E84	88284	88303
5-7-195	A85	**D85**	88277	88296	E85	88298	88317
5-7-340	A86	D86	88422	88441	E86	88443	88462
5-7-369	A87	D87	88451	88470	E87	88472	88491
5-7-378	A88	D88	88460	88479	E88	88481	88500
5-181-57	A89	D89	89374	89393	E89	89395	89414

5-181-127	A90	D90	89444	89463	E90	89465	89484
5-181-134	A91	D91	89451	89470	E91	89472	89491
5-181-321	A92	D92	89638	89657	E92	89659	89678
5-10-39	A93	**D93**	92740	92759	**E93**	92761	92780
5-10-302	A94	D94	93003	93022	E94	93024	93043
5-10-334	A95	D95	93035	93054	E95	93056	93075
5-11-158	A96	D96	93227	93246	**E96**	93248	93267
5-11-230	A97	D97	93299	93318	E97	93320	93339
5-11-234	A98	D98	93303	93322	E98	93324	93343
5-11-299	A99	D99	93368	93387	E99	93389	93408
5-11-304	A100	D100	93373	93392	E100	93394	93413
5-11-329	A101	D101	93398	93417	E101	93419	93438
5-12-56	A102	D102	93495	93514	E102	93516	93535
5-12-267	A103	D103	93706	93725	E103	93727	93746
5-13-145	A104	D104	93883	93902	E104	93904	93923
5-14-44	A105	D105	94150	94169	E105	94171	94190
5-14-93	A106	D106	94198	94217	E106	94219	94238
5-14-144	A107	D107	94249	94268	E107	94270	94289
5-14-165	A108	**D108**	94270	94289	E108	94291	94310
5-14-297	A109	D109	94402	94421	E109	94423	94442
5-14-307	A110	D110	94412	94431	E110	94433	94452
5-15-219	A111	**D111**	94700	94719	E111	94721	94740
5-16-157	A112	D112	94969	94988	E112	94990	95009
5-17-140	A113	D113	95241	95260	E113	95262	95281
5-18-51	A114	D114	95320	95339	E114	95341	95360
5-18-208	A115	**D115**	95477	95496	E115	95498	95517
5-300-238	A116	D116	95750	95769	E116	95771	95790
5-300-287	A117	D117	95799	95818	E117	95820	95839
5-262-49	A118	D118	96125	96144	E118	96146	96165
5-262-85	A119	D119	96161	96180	E119	96182	96201
5-262-254	A120	D120	96330	96349	E120	96351	96370
5-263-404	A121	D121	96931	96950	E121	96952	96971
5-265-244	A122	D122	97124	97143	E122	97145	97164
5-265-376	A123	D123	97256	97275	E123	97277	97296
99-7183-338	A124	**D124**	102247	102266	E124	102268	102287
99-7207-138	A125	D125	105917	105936	E125	105938	105957

Example 5

Association Study Between Prostate Cancer And The Biallelic Markers Of The *PCTA-1* Gene

Collection Of DNA Samples From Affected And Non-Affected Individuals

Affected population :

The positive trait followed in this association study was prostate cancer. Prostate cancer patients were recruited according to a combination of clinical, histological and biological

inclusion criteria. Clinical criteria can include rectal examination and prostate biopsies. Biological criteria can include PSA assays. The affected individuals were recorded as familial forms when at least two persons affected by prostate cancer have been diagnosed in the family. Remaining cases were classified as non-familial informative cases (at least two sibs of the case both aged over 50 years old are unaffected), or non-familial uniformative cases (no information about sibs over 50 years old is available). All affected individuals included in the statistical analysis of this patent were unrelated. Cases were also separated following the criteria of diagnosis age : early onset prostate cancer (under 65 years old) and late onset prostate cancer (65 years old or more).

Unaffected population :

Control individuals included in this study were checked for both the absence of all clinical and biological criteria defining the presence or the risk of prostate cancer (PSA < 4) (WO 96/21042), and for their age (aged 65 years old or more). All unaffected individuals included in the statistical analysis of this patent were unrelated.

The affected group was composed by 491 unrelated individuals, comprising:

- 197 familial cases among which 91 individuals were under 65 years old and 106 individuals were 65 years old or more; and

- 294 sporadic cases.

The unaffected group contained 313 individuals which were 65 years or older.

As used herein, the term "early onset cancer" refers to a cancer in which the individuals are under 65 years old.

Genotyping Of Affected And Control Individuals

The general strategy to perform the association studies was to individually scan the DNA samples from all individuals in each of the populations described above in order to establish the allele frequencies of the above described biallelic markers in each of these populations. More particularly, the biallelic markers used in the present association study are A2, A9, A15, A22, A24, A25, A26, A30, A34, A35, A36, A38, A41, A42, A44, A51, A52, A54, A55, A56, A57, A59, A60, A64, A73, A75, A76, A85, A93, A96, A108, A111, A115.

Allelic frequencies of the above-described biallelic markers in each population were determined by performing microsequencing reactions on amplified fragments obtained by genomic PCR performed on the DNA samples from each individual. Genomic PCR and microsequencing were performed as detailed above in examples 2 and 4 using the described PCR and microsequencing primers.

Association Study Between Prostate Cancer And The Biallelic Markers Of The PCTA-1 Gene

The alleles of two biallelic markers, namely (T) A30 and (T) A41, have been shown to be significantly associated to familial prostate cancer, more particularly early onset familial prostate cancer. Indeed, the allele T of the biallelic marker A30 showed a p-value of 1.08×10^{-2} for the early onset familial prostate cancer and of 3.39×10^{-2} for the familial prostate cancer. The allele T of the biallelic marker A41 presented a p-value of 4.04×10^{-2} for the early onset familial prostate cancer. These two markers could be then used in diagnostics.

Some other biallelic markers, namely A54, A55, A56, A57, A59, A60, A61, A85, A96, A108, A115, showed a moderate association. These biallelic markers are localized in the exons and introns of the PCTA-1 gene.

The inventors observed that all the *PCTA-1*-related biallelic markers were in linkage disequilibrium with each other in the controls individuals. In the familial cases of prostate cancer, the biallelic markers localized in the promoter did not show a linkage disequilibrium with those localized in exonic and intronic region of the *PCTA-1* gene and were not in linkage disequilibrium with each other. This lack of linkage disequilibrium for the promoter biallelic markers suggests that this region comprises a trait causing mutation and could explain the cases haplotypes.

A strong association has been observed between the allele A of the biallelic marker and sporadic cases of prostate cancer. This association is highly significant with a pvalue of 7.71×10^{-3}. The marker A2 can be then used in diagnostics.

Haplotype Frequency Analysis

One way of increasing the statistical power of individual markers, is by performing haplotype association analysis.

Haplotype analysis for association of *PCTA-1* markers and prostate cancer was performed by estimating the frequencies of all possible haplotypes comprising biallelic markers selected from the group consisting of A2, A9, A15, A22, A24, A25, A26, A30, A34, A35, A36, A38, A41, A42, A44, A51, A52, A54, A55, A56, A57, A59, A60, A64, A73, A75, A76, A85, A93, A96, A108, A111, A115 in the cases and control populations described in Example 5, and comparing these frequencies by means of a chi square statistical test (one degree of freedom). Haplotype estimations were performed by applying the Expectation-Maximization (EM) algorithm (Excoffier L & Slatkin M, 1995), using the EM-HAPLO program (Hawley ME, Pakstis AJ & Kidd KK, 1994).

Haplotype frequency analysis for familial cases of prostate cancer

The most significant haplotypes obtained with the familial cases of prostate cancer are shown in Table 5. These haplotypes comprise the biallelic markers A2, A30, A41, A55, A57, and 5-202/95.

The preferred two-markers haplotypes are described in Table 5 as H1 to H7 of PT2. The more preferred haplotype is the haplotype H1/PT2 and comprises the biallelic markers A30 (99-1572/440 allele T) and A41 (allele T). This haplotype presented a p-value of 1.1×10^{-4} and an odd-ratio of 1.67. Estimated haplotype frequencies were 57.2% in the cases and 44.4% in the controls.

The preferred three-markers haplotypes are described in Table 5 as H1, H2, H3, H7, H8, H9, H10, H11, and H12 of PT3. The more preferred haplotype is the haplotype H1/PT3 and comprises the biallelic markers A2 (allele A), A30 (99-1572/440 allele T) and A41 (allele T). This haplotype presented a p-value of 1.1×10^{-5} and an odd-ratio of 1.84. Estimated haplotype frequencies were 42.9% in the cases and 29% in the controls.

The preferred four-markers haplotypes are described in Table 5 as H1, H2, H4, H5, H7, H9, H16, H17, H18 and H19 of PT4.

In conclusion, most preferred haplotypes for the familial cases of prostate cancer comprise the biallelic markers A30 (99-1572/440 allele T) and/or A41 (allele T). Some other preferred haplotypes for the familial cases of prostate cancer comprise the biallelic marker A2 (allele A). Optionally, preferred haplotypes for the familial cases of prostate cancer comprise the biallelic markers A55 (allele C) and/or A57 (allele G). These haplotypes can be used in diagnostic of prostate cancer susceptibility.

Haplotype frequency analysis for sporadic cases of prostate cancer

The most significant haplotypes obtained with the sporadic cases of prostate cancer are shown in Table 6. These haplotypes comprise the biallelic markers A2, A30, A41, A55, A57, and 5-202/95.

The preferred two-markers haplotypes are described in Table 6 as H1 to H4, H6, and H7 of PT2. The first more preferred haplotype is the haplotype H1/PT2 and comprises the biallelic markers A2 (allele T) and A55 (allele T). This haplotype presented a p-value of 2.4×10^{-4} and an odd-ratio of 1.94. Estimated haplotype frequencies were 16.2% in the cases and 9% in the controls. The second more preferred haplotype is the haplotype H2/PT2 and comprises the biallelic markers A2 (allele T) and A57 (allele A). This haplotype presented a p-value of 5.3×10^{-4} and an odd-ratio of 1.84. Estimated haplotype frequencies were 16.3% in the cases and 9.5% in the controls.

The preferred three-markers haplotypes are described in Table 6 as H1, H2, H3, H4, H6, H7, and H8 of PT3. The more preferred haplotype is the haplotype H2/PT3 and comprises the biallelic markers A2 (allele T), A55 (allele T), and A57 (allele A). This haplotype presented a p-value of 2.3×10^{-3} and an odd-ratio of 1.75. Estimated haplotype frequencies were 15% in the cases and 9.2% in the controls.

The preferred four-markers haplotypes are described in Table 6 as H1, H2, H3, H4, and H6 of PT4.

In conclusion, most preferred haplotypes for the sporadic cases of prostate cancer comprise a biallelic marker selected from the group consisting of A2 (allele T), A55 (allele T), and A57 (allele A). Optionally, preferred haplotypes for the familial cases of prostate cancer comprise the biallelic markers A30 (allele T) and/or A41 (allele T). These haplotypes can be used in diagnostic of prostate cancer.

Summary of haplotype frequency analysis

The most preferred two- and three-biallelic markers haplotypes for the familial and sporadic prostate cancer are summarized in Table 7. These haplotypes can be used in diagnostic of prostate cancer susceptibility.

The statistical significance of the results obtained for the haplotype analysis was evaluated by a phenotypic permutation test reiterated 1000 or 10,000 times on a computer. For this computer simulation, data from the cases and control individuals were pooled and randomly allocated to two groups which contained the same number of individuals as the case-control populations used to produce the haplotype frequency analysis data. A haplotype analysis was then run on these artificial groups for the five haplotypes of the Table 7 which presented a strong association with prostate cancer. This experiment was reiterated 1000 times and the results are shown in Table 8.

The haplotypes 1 and 2 of the Table 7 are clearly associated with familial prostate cancer and more particularly with familial cases which were under 65 years and with >3caP familial cases. The permutation test clearly validate the statistical significance of the association between these haplotypes and familial prostate cancer since, among 1000 iterations, none of the obtained haplotypes had a p-value comparable to the one obtained for the haplotypes 1 and 2 of Table 7 for the familial cases, the familial cases under 65 years and the >3caP familial cases.

The haplotypes 3, 4, and 5 of the Table 7 are clearly associated with the sporadic prostate cancer. The permutation test clearly validate the statistical significance of the association between these haplotypes and sporadic prostate cancer since, among 1000 iterations,

less than 6 of the obtained haplotypes had a p-value comparable to the one obtained for the haplotypes 3, 4 and 5 of Table 7 for the sporadic cases. Moreover, among 1000 iterations, none of the obtained haplotypes had a p-value comparable to the one obtained for the haplotypes 3, 4 and 5 of Table 7 for the informative sporadic cases.

Attributable Risk

The attributable risk has been calculated as described in the "Evaluation of risk factors" of the part entitled "Statistic method". The results are disclosed in Table 9.

These results show that the preferred haplotypes disclosed in the present invention are highly significant for the prostate cancer. Indeed, 16.92 % of the sporadic prostate cancer cases carried the haplotype 4 of the Table 7 considering a dominant model which is the more relevant model for prostate cancer. Moreover, 60.77 % of the familial early onset prostate cancer cases carried the haplotype 1 of the Table 7 considering a dominant model.

Table 5: Haplotype frequency analysis for the familial cases of prostate cancer

			A2	A30	A41	A55	A57	A75	haplotype frequencies		Odds ratio	Pvalue (1df)
frequency %			67/67 (A)	72/66 (T)	75/71 (T)	72/68 (C)	72/69 (G)	95/95 (G)	cases	controls		
abs diff freq. all			0,1	6,4	4,2	3,8	3,4	0				
pvalue			7,5e-01	3,3e-02	1,4e-01	2,0e-01	2,5e-01	7,5e-01				
Cases/controls ↓												
H1	PT2	183/298		**T**	**T**				**0.572**	**0.444**	**1.67**	**1.1e-04**
H2		188/298		T			G		0.540	0.431	1.55	8.6e-04
H3		183/296		T		C			0.536	0.428	1.54	1.1e-03
H4		183/299	A		T				0.543	0.460	1.40	1.1e-02
H5		192/299	A	T					0.517	0.440	1.36	1.8e-02
H6		184/300		T				T	0.046	0.022	2.15	3.4e-02
H7		183/298	A			C			0.518	0.451	1.31	4.3e-02
H1	PT3	181/294	**A**	**T**	**T**				**0.429**	**0.290**	**1.84**	**1.1e-05**
H2		186/295	A	T			G		0.406	0.274	1.82	1.8e-05
H3		181/292	A	T		C			0.405	0.274	1.80	3.2e-05
H7		180/294		T	T	C			0.506	0.396	1.56	9.1e-04
H8		179/295		T			G	G	0.547	0.436	1.56	9.1e-04
H9		181/293		T		C		G	0.542	0.432	1.55	1.0e-03
H10		181/295		T	T			G	0.534	0.426	1.54	1.2e-03
H11		179/291		T		C	G		0.540	0.433	1.54	1.4e-03
H12		178/293		T	T		G		0.510	0.404	1.54	1.5e-03
H1	PT4	177/288	A	T		C	G		0.413	0.276	1.85	1.5e-05
H2		177/292	A	T			G	G	0.415	0.278	1.84	1.5e-05
H4		179/289	A	T		C		G	0.409	0.279	1.79	3.4e-05
H5		176/290	A	T	T		G		0.389	0.260	1.81	3.7e-05
H7		178/290	A	T	T	C			0.383	0.260	1.77	6.7e-05
H9		179/291	A	T	T			G	0.395	0.280	1.67	2.7e-04
H16		177/288		T		C	G	G	0.545	0.438	1.54	1.5e-03
H17		178/291		T	T	C		G	0.506	0.400	1.53	1.5e-03
H18		176/289		T	T	C	G		0.508	0.405	1.52	2.1e-03
H19		176/290		T	T		G	G	0.510	0.408	1.51	2.2e-03

1df refers to one degree of freedom.

Table 6: Haplotype frequency analysis for the sporadic cases of prostate cancer

			A2	A30	A41	A55	A57	A75	haplotype frequencies		Odds ratio	Pvalue (1df)
frequency %			60/67 (A)	64/66 (T)	73/71 (T)	64/68 (C)	65/69 (G)	94/95 (G)	cases	Controls		
abs diff freq. all.			-7,4	-2,0	2,7	-3,7	-3,9	-1				
pvalue			7,7e-03	4,3e-01	2,9e-01	1,6e-01	1,4e-01	3,4e-01				
Cases/controls ↓												
H1	PT2	281/298	**T**			**T**			**0.162**	**0.090**	**1.94**	**2.4e-04**
H2		282/301	**T**				**A**		**0.163**	**0.095**	**1.85**	**5.3e-04**
H3		282/301			T	T			0.140	0.083	1.79	2.1e-03
H4		283/298			T		A		0.136	0.083	1.74	3.6e-03
H6		283/299	T		T				0.317	0.246	1.42	7.3e-03
H7		279/300		T				T	0.045	0.022	2.08	3.0e-02
H1	PT3	278/295	T		T	T			0.083	0.037	2.33	1.1e-03
H2		277/294	**T**			**T**	**A**		**0.150**	**0.092**	**1.75**	**2.3e-03**
H3		279/295	T		T		A		0.081	0.040	2.12	3.4e-03
H4		278/294			T	T	A		0.134	0.082	1.75	3.8e-03
H6		277/295	T			T		G	0.126	0.076	1.76	4.7e-03
H7		277/293		T	T		A		0.091	0.048	1.96	4.7e-03
H8		275/294		T	T	T			0.093	0.051	1.91	5.5e-03
H1	PT4	273/290	T	T	T		A		0.046	0.010	4.76	2.0e-04
H2		271/290	T	T	T	T			0.044	0.010	4.54	3.9e-04
H3		274/291	T		T	T	A		0.078	0.038	2.15	3.6e-03
H4		274/292	T		T	T		G	0.053	0.021	2.57	4.4e-03
H6		272/289		T	T	T	A		0.090	0.048	1.95	5.5e-03

Table 7: Haplotype frequency analysis of the preferred haplotypes

		HAPLOTYPE						Pvalue haplo. Frequency % (cases vs controls)	
	MARKERS		A2	A30	A41	A55	A57	familial cases vs controls	sporadic cases vs controls
FAMILIAL CASES HAPLOTYPES	**PT2**	**haplotype 1**		**T**	**T**			**1e-04 (57/44)**	6e-01 (45/44)
	PT3	**haplotype 2**	**A**	**T**	**T**			**1e-05 (43/29)**	2e-01 (26/29)
SPORADICS CASES HAPLOTYPES	**PT2**	**haplotype 3**	**T**				**A**	4e-01 (11/10)	**5e-04 (16/10)**
		haplotype 4	**T**			**T**		3e-01 (11/9)	**2e-04 (16/9)**
	PT3	**haplotype 5**	**T**			**T**	**A**	3e-01 (11/9)	**2e-03 (15/9)**

Table 8: Haplotype frequency analysis with permutation test results

SAMPLES	number cases/ controls	haplotype frequency		Odds ratio	Pvalue (1df)	PERMUTATIONS TEST Iter /nb of Iter.
		cases	controls			
HAPLOTYPE 1 of Table 7						
cases vs controls	463/298	0.501	0.444	1.26	2.8e-02	30/1000
cases (<=65 years) vs controls	176/298	0.546	0.444	1.51	2.3e-03	3/1000
cases (>65 years) vs controls	283/298	0.467	0.444	1.10	4.0e-01	273/1000
sporadic cases vs controls	280/298	0.455	0.444	1.04	6.5e-01	572/1000
sporadic cases (<=65 years) vs controls	89/298	0.454	0.444	1.04	7.5e-01	696/1000
sporadic cases (>65 years) vs controls	187/298	0.450	0.444	1.02	7.5e-01	771/1000
sporadic informatif vs controls	67/298	0.434	0.444	0.96	7.5e-01	699/1000
familial cases vs controls	183/298	0.572	0.444	1.67	1.1e-04	0/1000
familial cases (<=65 years) vs controls	87/298	**0.646**	**0.444**	**2.28**	**2.7e-06**	0/1000
familial cases (>65 years) vs controls	96/298	0.501	0.444	1.25	1.7e-01	103/1000
familial cases (>=3caP) vs controls	82/298	0.588	0.444	1.79	1.1e-03	0/1000
HAPLOTYPE 2 of Table 7						
cases vs controls	457/294	0.325	0.290	1.18	1.4e-01	127/1000
cases (<=65 years) vs controls	174/294	0.362	0.290	1.39	2.1e-02	24/1000
cases (>65 years) vs controls	279/294	0.297	0.290	1.03	7.5e-01	770/1000
sporadic cases vs controls	276/294	0.257	0.290	0.85	2.1e-01	176/1000
sporadic cases (<=65 years) vs controls	88/294	0.229	0.290	0.73	1.1e-01	99/1000
sporadic cases (>65 years) vs controls	184/294	0.265	0.290	0.88	4.0e-01	351/1000
sporadic informatif vs controls	67/294	0.176	0.290	0.52	6.9e-03	9/1000
familial cases vs controls	181/294	0.429	0.290	1.84	1.1e-05	0/1000
familial cases (<=65 years) vs controls	86/294	**0.501**	**0.290**	2.46	**2.5e-07**	0/1000
familial cases (>65 years) vs controls	95/294	0.365	0.290	1.41	4.8e-02	48/1000
familial cases (>=3caP) vs controls	82/294	0.467	0.290	2.14	2.0e-05	0/1000
HAPLOTYPE 3 of Table 7						
cases vs controls	470/301	0.143	0.095	1.58	5.8e-03	15/1000
cases (<=65 years) vs controls	175/301	0.132	0.095	1.44	7.8e-02	96/1000
cases (>65 years) vs controls	292/301	0.145	0.095	1.61	8.2e-03	14/1000
sporadic cases vs controls	282/301	**0.163**	**0.095**	1.85	**5.3e-04**	4/1000
sporadic cases (<=65 years) vs controls	90/301	0.163	0.095	1.85	1.1e-02	11/1000
sporadic cases (>65 years) vs controls	189/301	0.158	0.095	1.77	3.4e-03	10/1000
sporadic informatif vs controls	70/301	**0.221**	**0.095**	2.69	**3.4e-05**	0/1000
familial cases vs controls	188/301	0.110	0.095	1.17	4.4e-01	487/1000
familial cases (<=65 years) vs controls	85/301	0.096	0.095	1.00	7.5e-01	991/1000
familial cases (>65 years) vs controls	103/301	0.121	0.095	1.31	2.7e-01	317/1000
familial cases (>=3caP) vs controls	83/301	0.074	0.095	0.76	3.7e-01	462/1000
HAPLOTYPE 4 of Table 7						
cases vs controls	464/298	0.143	0.090	1.68	2.2e-03	7/1000
cases (<=65 years) vs controls	174/298	0.135	0.090	1.57	3.0e-02	47/1000
cases (>65 years) vs controls	286/298	0.145	0.090	1.70	3.8e-03	9/1000

sporadic cases vs controls	281/298	**0.162**	**0.090**	1.94	**2.4e-04**	2/1000
sporadic cases (<=65 years) vs controls	88/298	0.165	0.090	2.00	4.7e-03	17/1000
sporadic cases (>65 years) vs controls	189/298	0.156	0.090	1.87	1.7e-03	4/1000
sporadic informatif vs controls	69/298	**0.223**	**0.090**	2.89	**1.1e-05**	0/1000
familial cases vs controls	183/298	0.110	0.090	1.25	2.9e-01	318/1000
familial cases (<=65 years) vs controls	86/298	0.100	0.090	1.12	6.5e-01	726/1000
familial cases (>65 years) vs controls	97/298	0.120	0.090	1.37	2.2e-01	271/1000
familial cases (>=3caP) vs controls	81/298	0.084	0.090	0.93	7.5e-01	839/1000
HAPLOTYPE 5 of Table 7						
cases vs controls	456/294	0.136	0.092	1.56	9.1e-03	14/1000
cases (<=65 years) vs controls	171/294	0.131	0.092	1.48	6.5e-02	80/1000
cases (>65 years) vs controls	282/294	0.136	0.092	1.55	1.8e-02	30/1000
sporadic cases vs controls	277/294	**0.150**	**0.092**	1.75	**2.3e-03**	6/1000
sporadic cases (<=65 years) vs controls	88/294	0.155	0.092	1.81	1.7e-02	27/1000
sporadic cases (>65 years) vs controls	186/294	0.142	0.092	1.64	1.5e-02	34/1000
sporadic informatif vs controls	69/294	**0.226**	**0.092**	2.89	**1.0e-05**	0/1000
familial cases vs controls	179/294	0.112	0.092	1.24	3.2e-01	354/1000
familial cases (<=65 years) vs controls	83/294	0.102	0.092	1.12	6.5e-01	733/1000
familial cases (>65 years) vs controls	96/294	0.121	0.092	1.36	2.4e-01	262/1000
familial cases (>=3caP) vs controls	79/294	0.082	0.092	0.88	6.5e-01	749/1000

Familial forms in which at least three persons are affected by prostate cancer in the family are described in the present application as >3CaP. Sporadic cases were classified as informative sporadic cases when at least two sibs of the case both aged over 50 years old are unaffected.

Table 9: Attributable risk for prostate cancer

	Sample sizes cases vs controls	Estimating of haplotype frequency			**Dominant Model**		**Recessif Model**	
		cases	Controls (unaffected)	Random controls (French)	**Carriers frequency (cases vs controls)**	**Attribu-table Risk %**	**Carriers frequency (cases vs controls)**	**Attribu-table Risk %**
Haplotype 4 of Table 7	281 vs 298	16.2%	9 %	10.3 %	30% vs 17%	**16,92**	3% vs 1%	**2,38**
Haplotype 1 of Table 7	87 vs 298	64.6%	44 %	48 %	88% vs 69%	**60,77**	42% vs 20%	**30,63**

CARRIER: Individual carrying the haplotype

ODD RATIO of Carrier (OR): Carrier of cases * (1-Carrier of controls) / Carrier of controls * (1-Carrier of cases)

ATTRIBUTABLE RISK (RR): Carrier of Randoms controls * (OR -1) / (Carrier of Randoms controls * (OR -1) + 1)

(ref: Epidémiologie - Principes et méthodes quantitatives - J. Bouyer, D. Hémon, S. Cordier 1995)

Example 6

Mouse PCTA-1 protein

The inventors have cloned a cDNA molecule encoding a mouse homologue of the PCTA-1 protein (SEQ ID No 8). The deduce amino acid sequence is provided in SEQ ID No 9. Figures 7A-D show alignments between the human and mouse PCTA-1 protein sequences of the inventions, as well as that of GenBank L78132. It shows an 80 % homology between the human and mouse homologues.

Further comparisons between these mouse and human cDNA and protein sequences, taking into consideration the position of significant polymorphisms in relation with potentially conserved motifs, should allow the person skilled in the art to identify regions of specific physiological interest, in the design of suitable vaccine or therapeutic candidates. Two galactoside binding sites shown in figures 7A-D are of special interest. These sites are conserved among the PCTA-1 proteins and the galectins, and seem to be involved in the cell-cell and cell-matrix interactions which are of high relevance to cancer. Two other sites, HFNPRF and VVCN, are also highly conserved among all these proteins.

Example 7

Preparation Of Antibody Compositions To A PCTA-1 Protein

Substantially pure protein or polypeptide is isolated from transfected or transformed cells containing an expression vector encoding a PCTA-1 protein or a portion thereof. The concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the protein can then be prepared as follows:

A. Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes in a PCTA-1 protein or a portion thereof can be prepared from murine hybridomas according to the classical method of Kohler et al. (1975) or derivative methods thereof. Also see Harlow et al. 1988.

Briefly, a mouse is repetitively inoculated with a few micrograms of a PCTA-1 protein or a portion thereof over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as

ELISA, as originally described by Engvall, (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis et al.

B. Polyclonal Antibody Production by Immunization

Polyclonal antiserum containing antibodies to heterogeneous epitopes in a PCTA-protein or a portion thereof can be prepared by immunizing suitable non-human animal with this PCTA-1 protein or a portion thereof, which can be unmodified or modified to enhance immunogenicity. A suitable non-human animal is preferably a non-human mammal is selected, usually a mouse, rat, rabbit, goat, or horse. Alternatively, a crude preparation which has been enriched for the PCTA-1 concentration can be used to generate antibodies. Such proteins, fragments or preparations are introduced into the non-human mammal in the presence of an appropriate adjuvant (e.g. aluminum hydroxide, RIBI, etc.) which is known in the art. In addition the protein, fragment or preparation can be pretreated with an agent which will increase antigenicity, such agents are known in the art and include, for example, methylated bovine serum albumin (mBSA), bovine serum albumin (BSA), Hepatitis B surface antigen, and keyhole limpet hemocyanin (KLH). Serum from the immunized animal is collected, treated and tested according to known procedures. If the serum contains polyclonal antibodies to undesired epitopes, the polyclonal antibodies can be purified by immunoaffinity chromatography.

Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987). An effective immunization protocol for rabbits can be found in Vaitukaitis, et al. (1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. et al., (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, (1980).

Antibody preparations prepared according to either the monoclonal or the polyclonal protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively

to identify the presence of antigen in a biological sample. The antibodies may also be used in therapeutic compositions for killing cells expressing the protein or reducing the levels of the protein in the body.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein by the one skilled in the art without departing from the spirit and scope of the invention. Accordingly, the scope of this invention is intended to be defined only by reference to the appended claims.

References

The following references are cited herein and are incorporated herein by reference in their entireties:

Abbondanzo SJ et al., 1993, Methods in Enzymology, Academic Press, New York, pp 803-823

Ajioka R.S. et al., *Am. J. Hum. Genet.*, 60:1439-1447, 1997

Altschul et al., 1990, J. Mol. Biol. 215(3):403-410

Altschul et al., 1993, Nature Genetics 3:266-272

Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402

Anton M. et al., 1995, J. Virol., **69** : 4600-4606

Araki K et al. (1995) *Proc. Natl. Acad. Sci. USA.* 92(1):160-4.

Aszódi et al., Proteins:Structure, Function, and Genetics, Supplement 1:38-42 (1997)

Ausubel et al. (1989)Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Baubonis W. (1993) *Nucleic Acids Res.* 21(9):2025-9.

Beaucage et al., *Tetrahedron Lett* 1981, **22**: 1859-1862

Bram RJ et al., 1993, Mol. Cell Biol., **13** : 4760-4769

Brown EL, Belagaje R, Ryan MJ, Khorana HG, *Methods Enzymol* 1979;**68**:109-151

Brutlag et al. Comp. App. Biosci. 6:237-245, 1990

Bush et al., 1997, J. Chromatogr., 777 : 311-328.

Chai H. et al. (1993) *Biotechnol. Appl. Biochem.*18:259-273.

Chee et al. (1996) *Science.* 274:610-614.

Chen and Kwok *Nucleic Acids Research* 25:347-353 1997

Chen et al. (1987) *Mol. Cell. Biol.* 7:2745-2752.

Chen et al. *Proc. Natl. Acad. Sci. USA* 94/20 10756-10761,1997

Cho RJ et al., 1998, Proc. Natl. Acad. Sci. USA, **95**(7) : 3752-3757.

Chou J.Y., 1989, Mol. Endocrinol., **3**: 1511-1514.

Clark A.G. (1990) *Mol. Biol. Evol.* 7:111-122.

Coles R, Caswell R, Rubinsztein DC, *Hum Mol Genet* 1998;**7**:791-800

Compton J. (1991) *Nature.* 350(6313):91-92.

Davis L.G., M.D. Dibner, and J.F. Battey, Basic Methods in Molecular Biology, ed., Elsevier Press, NY, 1986

Dempster et al., (1977) *J. R. Stat. Soc.*, 39B:1-38.

Dent DS & Latchman DS (1993) The DNA mobility shift assay. In: *Transcription Factors: A Practical Approach* (Latchman DS, ed.) pp1-26. Oxford: IRL Press

Dignam JD, et al, *Nucleic Acids Res.* 1983 Mar 11; **11**(5): 1475-1489.

Doucas V, et al, *EMBO J.* 1991 Aug; **10**(8): 2237-2245

Dynan WS, Tjian R, *Cell* 1983;**35**:79-87

Edwards et Leatherbarrow, *Analytical Biochemistry*, **246**, 1-6 (1997)

Engvall, E., Meth. Enzymol. 70:419 (1980)

Excoffier L. and Slatkin M. (1995) *Mol. Biol. Evol.*, 12(5): 921-927.

Feldman and Steg, 1996, Medecine/Sciences, synthese, 12:47-55

Felici F., 1991, J. Mol. Biol., Vol. 222:301-310

Fields and Song, 1989, Nature, **340** : 245-246

Fisher, D., Chap. 42 in: Manual of Clinical Immunology, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980)

Flotte et al. (1992) *Am. J. Respir. Cell Mol. Biol.* 7:349-356.

Fodor et al. (1991) *Science* 251:767-777.

Fraley et al. (1979) *Proc. Natl. Acad. Sci. USA.* 76:3348-3352.

Fried M, Crothers DM, *Nucleic Acids Res* 1981;**9**:6505-6525

Fromont-Racine M. et al., 1997, Nature Genetics, **16**(3) : 277-282.

Fuller S. A. et al. (1996) *Immunology in Current Protocols in Molecular Biology*, Ausubel et al.Eds, John Wiley & Sons, Inc., USA.

Furth P.A. et al. (1994) *Proc. Natl. Acad. Sci USA.* 91:9302-9306.

Galas DJ, Schmitz A, *Nucleic Acids Res* 1978;**5**:3157-3170

Garner MM, Revzin A, *Nucleic Acids Res* 1981;**9**:3047-3060

Geysen H. Mario et al. 1984. Proc. Natl. Acad. Sci. U.S.A. 81:3998-4002

Ghosh and Bacchawat, 1991, *Targeting of liposomes to hepatocytes*, IN: *Liver Diseases, Targeted diagnosis and therapy using specific rceptors and ligands*. Wu et al. Eds., Marcel Dekeker, New York, pp. 87-104.

Gonnet et al., 1992, Science 256:1443-1445

Gopal (1985) *Mol. Cell. Biol.*, 5:1188-1190.

Gossen M. et al. (1992) *Proc. Natl. Acad. Sci. USA.* 89:5547-5551.

Gossen M. et al. (1995) *Science.* 268:1766-1769.

Graham et al. (1973) *Virology* 52:456-457.

Green et al., *Ann. Rev. Biochem.* **55**:569-597 (1986)

Griffin et al. *Science* **245**:967-971 (1989)

Grompe, M. (1993) *Nature Genetics.* 5:111-117.

Grompe, M. et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5855-5892.

Gu H. et al. (1993) *Cell* 73:1155-1164.

Gu H. et al. (1994) *Science* 265:103-106.

Guatelli J C et al. *Proc. Natl. Acad. Sci. USA.* 35:273-286.

Hacia JG, Brody LC, Chee MS, Fodor SP, Collins FS, *Nat Genet* 1996;**14**(4):441-447

Haff L. A. and Smirnov I. P. (1997) *Genome Research*, 7:378-388.

Hames B.D. and Higgins S.J. (1985*) Nucleic Acid Hybridization: A Practical Approach.* Hames and Higgins Ed., IRL Press, Oxford.

Harju L, Weber T, Alexandrova L, Lukin M, Ranki M, Jalanko A, *Clin Chem* 1993;**39**(11Pt 1):2282-2287

Harland et al. (1985) *J. Cell. Biol.* 101:1094-1095.

Harlow, E., and D. Lane. 1988. Antibodies A Laboratory Manual. Cold Spring Harbor Laboratory. pp. 53-242

Harper JW et al., 1993, Cell, **75** : 805-816

Hawley M.E. et al. (1994) *Am. J. Phys. Anthropol.* 18:104.

Henikoff and Henikoff, 1993, Proteins 17:49-61

Higgins et al., 1996, Methods Enzymol. 266:383-402

Hillier L. and Green P. *Methods Appl.,* 1991, **1**: 124-8.

Hoess et al. (1986) *Nucleic Acids Res.* 14:2287-2300.

Huang L. et al. (1996) *Cancer Res* 56(5):1137-1141.

Huygen et al. (1996) *Nature Medicine.* 2(8):893-898.

Izant JG, Weintraub H, *Cell* 1984 Apr;**36**(4):1007-15

Julan et al. (1992) *J. Gen. Virol.* 73:3251-3255.

Kanegae Y. et al., *Nucl. Acids Res.* 23:3816-3821.

Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2267-2268

Khoury J. et al., *Fundamentals of Genetic Epidemiology, Oxford University Press, NY,* 1993

Kim U-J. et al. (1996) *Genomics* 34:213-218.

Klein et al. (1987) *Nature.* 327:70-73.

Kohler, G. and Milstein, C., Nature 256:495 (1975)

Koller et al. (1992) *Annu. Rev. Immunol.* 10:705-730.

Kozal MJ, Shah N, Shen N, Yang R, Fucini R, Merigan TC, Richman DD, Morris D, Hubbell E, Chee M, Lander and Schork, *Science,* 265, 2037-2048, 1994

Landegren U. et al. (1998) *Genome Research*, 8:769-776.

Lange K. (1997) *Mathematical and Statistical Methods for Genetic Analysis.* Springer, New York.

Lenhard T. et al. (1996) *Gene.* 169:187-190.

Linton M.F. et al. (1993) *J. Clin. Invest.* 92:3029-3037.

Livak et al., *Nature Genetics*, 9:341-342, 1995

Livak KJ, Hainer JW, *Hum Mutat* 1994;**3**(4):379-385

Lockhart et al. *Nature Biotechnology* **14**: 1675-1680, 1996

Lucas A.H., 1994, In : Development and Clinical Uses of Haempophilus b Conjugate;

Manley JL, et al, *Proc Natl Acad Sci U S A*. 1980 Jul; **77**(7): 3855-3859

Mansour S.L. et al. (1988) *Nature*. 336:348-352.

Marshall R. L. et al. (1994) *PCR Methods and Applications*. 4:80-84.

Maxam AM, Gilbert W, *Methods Enzymol* 1980;**65**:499-560

McCormick et al. (1994) *Genet. Anal. Tech. Appl.* 11:158-164.

McLaughlin B.A. et al. (1996) *Am. J. Hum. Genet.* 59:561-569.

Mizokami A, Yeh SY, Chang C, *Mol Endocrinol* 1994;**8**:77-88

Morton N.E., *Am.J. Hum.Genet.*, 7:277-318, 1955

Muller MM, Schreiber E, Schaffner W, Matthias P, *Nucleic Acids Res* 1989;**17**:6420

Muzyczka et al. (1992) *Curr. Topics in Micro. and Immunol.* 158:97-129.

Nada S. et al. (1993) *Cell* 73:1125-1135.

Nagy A. et al., 1993, Proc. Natl. Acad. Sci. USA, **90**: 8424-8428.

Narang SA, Hsiung HM, Brousseau R, *Methods Enzymol* 1979;**68**:90-98

Neda et al. (1991) *J. Biol. Chem.* 266:14143-14146.

Newton et al. (1989) *Nucleic Acids Res.* 17:2503-2516.

Nickerson D.A. et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:8923-8927.

Nicolau C. et al., 1987, Methods Enzymol., **149**:157-76.

Nicolau et al. (1982) *Biochim. Biophys. Acta.* 721:185-190.

Nihei et al, *Genes Chromosomes Cancer* 1995;**14**:112-119

Nyren P, Pettersson B, Uhlen M, *Anal Biochem* 1993;**208**(1):171-175

O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W. H. Freeman and Co., New York.

Ohno et al. (1994) *Science.* 265:781-784.

Oldenburg K.R. et al., 1992, Proc. Natl. Acad. Sci., **89**:5393-5397.

Orita et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.*86: 2776-2770.

Ott J., *Analysis of Human Genetic Linkage, John Hopkins University Press, Baltimore,* 1991

Ouchterlony, O. et al., Chap. 19 in: Handbook of Experimental Immunology D. Wier (ed) Blackwell (1973)

Parmley and Smith, Gene, 1988, **73**:305-318

Pastinen et al., *Genome Research* 1997; 7:606-614

Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85(8):2444-2448

Pease S. ans William R.S., 1990, Exp. Cell. Res., **190**: 209-211.

Perlin et al. (1994) *Am. J. Hum. Genet.* 55:777-787.

Peterson et al., 1993, Proc. Natl. Acad. Sci. USA, **90** : 7593-7597.

Pietu et al. *Genome Research* **6**:492-503, 1996

Potter et al. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81(22):7161-7165.

Ramunsen et al., 1997, Electrophoresis, **18** : 588-598.

Reid L.H. et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:4299-4303.

Risch, N. and Merikangas, K. (*Science*, 273:1516-1517, 1996

Robertson E., 1987, Embryo-derived stem cell lines. In: E.J. Robertson Ed. *Teratocarcinomas and embrionic stem cells: a practical approach.* IRL Press, Oxford, pp. 71.

Rossi et al., *Pharmacol. Ther.* **50**:245-254, (1991)

Roth J.A. et al. (1996) *Nature Medicine.* 2(9):985-991.

Roux et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:9079-9083.

Ruano et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:6296-6300.

Sambrook, J., Fritsch, E.F., and T. Maniatis. (1989) *Molecular Cloning: A Laboratory Manual.* 2ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Samson M, et al. (1996) *Nature*, 382(6593):722-725.

Samulski et al. (1989) *J. Virol.* 63:3822-3828.

Sanchez-Pescador R. (1988) *J. Clin. Microbiol.* 26(10):1934-1938.

Sarkar, G. and Sommer S.S. (1991) *Biotechniques.*

Sauer B. et al. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:5166-5170.

Saunders AM, et al, *Neurology* 1993;**43;**1467-1472

Schaid D.J. et al., *Genet. Epidemiol.*,13:423-450, 1996

Schedl A. et al., 1993a, Nature, **362**: 258-261.

Schedl et al., 1993b, Nucleic Acids Res., **21**: 4783-4787.

Schena et al. *Science* **270**:467-470, 1995

Schena et al., 1996, Proc Natl Acad Sci U S A,.**93**(20):10614-10619.

Schneider et al.(1997) *Arlequin: A Software For Population Genetics Data Analysis.* University of Geneva.

Schreiber E, Matthias P, Muller MM, Schaffner W, *Nucleic Acids Res* 1989 ;**17**:6419

Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure, Washington: National Biomedical Research Foundation

Sczakiel G. et al. (1995) *Trends Microbiol.* 3(6):213-217.

Shay J.W. et al., 1991, Biochem. Biophys. Acta, **1072**: 1-7.

Sheffield, V.C. et al. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 49:699-706.

Shizuya et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:8794-8797.

Shoemaker DD, et al., *Nat Genet* 1996;**14**(4):450-456

Siebenlist U, Gilbert W, *Proc Natl Acad Sci U S A* 1980;**77**:122-126

Smith (1957) *Ann. Hum. Genet.* 21:254-276.

Smith et al. (1983) *Mol. Cell. Biol.* 3:2156-2165.

Sosnowski RG, et al., *Proc Natl Acad Sci U S A* 1997;**94**:1119-1123

Sowdhamini et al., Protein Engineering 10:207, 215 (1997)

Spielmann S. and Ewens W.J., *Am. J. Hum. Genet.*, 62:450-458, 1998

Spielmann S. et al., *Am. J. Hum. Genet.*, 52:506-516, 1993

Sternberg N.L. (1994) *Mamm. Genome.* 5:397-404.

Strittmatter WJ, et al., *Proc Natl Acad Sci U S A* 1993 ;**90**:1977-1981

Stryer, L., *Biochemistry,* 4th edition, 1995

Syvanen AC, *Clin Chim Acta* 1994;**226**(2):225-236

Szabo A. et al. *Curr Opin Struct Biol* **5**, 699-705 (1995)

Szabo et al., 1995, Curr Opin Struct Biol., **5**(5):699-705

Tacson et al. (1996) *Nature Medicine.* 2(8):888-892.

Te Riele et al. (1990) Nature. 348:649-651.

Terwilliger J.D. and Ott J., *Handbook of Human Genetic Linkage, John Hopkins University Press, London,* 1994

Thomas K.R. et al. (1986) *Cell.* 44:419-428.

Thomas K.R. et al. (1987) *Cell.* 51:503-512.

Thompson et al., 1994, Nucleic Acids Res. 22(2):4673-4680

Tur-Kaspa et al. (1986) *Mol. Cell. Biol.* 6:716-718.

Tyagi et al. (1998) *Nature Biotechnology.* 16:49-53.

Urdea M.S. (1988) *Nucleic Acids Research.* 11:4937-4957.

Urdea M.S. et al.(1991) *Nucleic Acids Symp. Ser.* 24:197-200.

Vaitukaitis, J. et al. J. Clin. Endocrinol. Metab. 33:988-991 (1971)

Valadon P., et al., 1996, J. Mol. Biol., **261**:11-22.

Van der Lugt et al. (1991) *Gene.* 105:263-267.

Vlasak R. et al. (1983) *Eur. J. Biochem.* 135:123-126.

Wabiko et al. (1986) *DNA.*5(4):305-314.

Walker et al. (1996) *Clin. Chem.* 42:9-13.

Wang et al., 1997, Chromatographia, **44** : 205-208.

Weir, B.S. (1996) *Genetic data Analysis II: Methods for Discrete population genetic Data, Sinauer Assoc., Inc., Sunderland, MA, U.S.A.*

Westerink M.A.J., 1995, Proc. Natl. Acad. Sci., **92**:4021-4025

White, M.B. et al. (1992) *Genomics.* 12:301-306.

White, M.B. et al. (1997) *Genomics.* 12:301-306.

Wong et al. (1980) *Gene.* 10:87-94.

Wood S.A. et al., 1993, Proc. Natl. Acad. Sci. USA, **90**: 4582-4585.

Wu and Wu (1987) *J. Biol. Chem.* 262:4429-4432.

Wu and Wu (1988) *Biochemistry.* 27:887-892.

Wu et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:2757.

Yagi T. et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:9918-9922.

Zhao et al., *Am. J. Hum. Genet.*, 63:225-240, 1998

Zou Y. R. et al. (1994) *Curr. Biol.* 4:1099-1103.